Effects of ocean acidification on the physiology of different life stages of *Hyas araneus*

Auswirkungen der Ozeanversauerung auf die Physiologie verschiedener Lebensstadien von *Hyas araneus*

Dissertation
Zur Erlangung des akademischen Grades
- Dr. rer. nat. –

dem Fachbereich 2 Biologie / Chemie
der Universität Bremen
vorgelegt von

Melanie Schiffer
Diplom-Biologin

Bremen 2013

Gutachter

1. Gutachter: Prof. Dr. H. O. Pörtner, Universität Bremen
   Alfred-Wegener-Institut Helmholtz-Zentrum für
   Polar- und Meeresforschung, Integrative Ecophysiology
   Am Handelshafen 12, Bremen 27570

2. Gutachter: PD. Dr. H. Auel, Universität Bremen
   Fachbereich II/Marine Zoologie
   Loebener Strasse, Bremen 28359

Table of contents

List of abbreviations

AMR	active metabolic rate
CA	carbonic anhydrase
CCO_2	inorganic carbon content
CO_3^{2-}	carbonate
DIC	dissolved inorganic carbon
HCO_3	bicarbonate
H_2CO_3	carbon acid
HSP	heat shock protein
KF	potassium fluoride
MR	metabolic rate
NaK	sodium potassium ATPase
NBC	sodium bicarbonate co-transporter
NHE	sodium proton exchanger
NTA	nitrilotriacetic acid
PVF	perivitelline fluid
pH_e	extracellular pH
PNR	point of no return
PRS	point of reserve saturation
Q	quantities
SMR	standard metabolic rate
T_c	critical temperature
T_d	denaturation temperature
T_p	pejus temperature

Summary

Anthropogenic CO_2 emissions and global warming threaten marine ecosystems by increasing water temperature and decreasing seawater pH. Studying the effects of high seawater P_{CO_2} on marine organism physiology is a crucial prerequisite to estimate ecosystem-level responses to climate change. Species most at risk might be the poor osmoregulators due to limited compensatory capacities of acid-base disturbances during hypercapnic exposure. The present thesis sets out to investigate the long-term physiological effects of elevated seawater P_{CO_2} and temperature on different life stages of the osmoconforming spider crab *Hyas araneus*. Pelagic and benthic life stages of *H. araneus* occupy habitats with different environmental characteristics and might be differentially affected by ocean acidification and warming.

The goal of the present study was to develop an integrative view how the acid-base status and the capacity to regulate it affects the animal´s energy budget. Extracellular pH of adult crabs decreased with increasing seawater CO_2 (1000 – 3000 µatm), but was compensated to various degrees by an increase in bicarbonate. While mRNA expression of several genes important for acid-base regulation regulation was not affected by high seawater P_{CO_2} in adults and larvae of *Hyas araneus*, mRNA expression of genes involved in mitochondrial energy metabolism was increased in larvae in response to elevated P_{CO_2}. These elevated transcript levels of mitochondrial genes could point to mitochondrial acclimation towards elevated seawater P_{CO_2}. Exposure to elevated seawater CO_2 did not affect the oxygen consumption of larval and adult *H. araneus*. The effects of elevated seawater P_{CO_2} on the acid-base status and metabolism did not compromise larval and adult survival.

Consequently, studies on early developmental and potentially more sensitive life stages were conducted. The impact of high seawater CO_2 on development, growth and survival of the first larval stage (zoea) with different pre-hatch history (high CO_2 during pre-and post-hatching phase vs. normocapnic CO_2 during the pre-hatching phase and high CO_2 during the post-hatching phase) was examined. Effects of post-hatching exposure to elevated seawater P_{CO_2} on oxygen consumption and growth in developing zoea I larvae of *H. araneus* were small, developmental duration and survival remained unaffected. In contrast, zoea I larvae pre-exposed to high seawater P_{CO_2} during maternal care showed extremely high mortality rates and severe developmental delay due to potentially hypercapnia-induced changes in the early larval energy budget leading to insufficient growth increments.

The high mortality rates observed in pre-exposed zoea I larvae were not due to changes in the egg fluid pH and P_{CO_2}. Embryos might also not be affected by elevated seawater P_{CO_2} through less oxygen supply by the females. In fact, hypercapnic exposure leads to altered female brooding behaviour with more effort spent in egg mass ventilation. This might have crucial consequences for the female's energy budget.

In order to address synergistic effects of elevated seawater CO_2 and temperature on larvae of *H. araneus*, the temperature tolerance window of zoea and megalopa larvae exposed to different seawater P_{CO_2} was determined. The upper thermal tolerance limit was lowered at high CO_2 in zoea larvae. This suggests that high CO_2 and elevated temperatures act synergistically on physiological mechanisms of the larvae. A comparative genetic approach revealed an increased transcriptomic response to acute heat shock with elevated transcript levels of heat shock proteins in larvae exposed to elevated seawater P_{CO_2}.

The experimental results from this thesis indicate that osmoconforming crustacean species are comparatively tolerant towards ocean acidification. Nonetheless, severe impacts of elevated seawater P_{CO_2} could be detected in certain early life cycle stages of *H. araneus*. Transition between pre-hatch embryos and post-hatch larvae seem to be a critical bottleneck within the life cycle of *H. araneus*. Carry-over effects between life stages and/or CO_2 induced disturbances of the transition phases from one to the next stage have the potential to severely impact species survival.

Zusammenfassung

Anthropogene CO_2 Emissionen und die globale Erwärmung gefährden marine Ökosysteme aufgrund von sowohl steigenden Temperaturen des Meerwassers als auch sinkendem Meerwasser pH. Studien, die sich mit den Auswirkungen erhöhter Seewasser CO_2-Konzentrationen auf die Physiologie mariner Organismen beschäftigen, sind eine wichtige Grundvoraussetzung, um die Antwort von Ökosystemen auf den Klimawandel abzuschätzen zu können. Aufgrund ihrer limitierten Kapazität Säure-Base Störungen zu kompensieren, könnten schwache Osmoregulatoren die am stärksten gefährdeten Arten sein. Die vorgelegte Arbeit setzt sich mit den Langzeitauswirkungen eines erhöhten Seewasser CO_2 Partialdrucks und erhöhten Temperaturen auf die Physiologie verschiedener Lebensstadien der osmokonformen Seespinne *Hyas araneus* auseinander. Pelagische und benthische Lebensstadien von *H. araneus* sind in ihren Habitaten unterschiedlich stark schwankenden Umwelteinflüssen ausgesetzt. Ozeanversauerung und Erwärmung könnten daher einen unterschiedlich starken Einfluss auf die verschiedenen Lebensstadien haben.

Ein Ziel der vorliegenden Studie war es zu untersuchen wie der Säure-Base Haushalt und die Kapazität diesen zu regulieren, das Energiebudget von Tieren beeinflusst. Es konnte gezeigt werden, dass der extrazelluläre pH in adulten Krebsen mit steigender Seewasser CO_2-Konzentration (1000 – 3000 µatm) sank, jedoch teilweise durch eine Akkumulation von Bikarbonate kompensiert werden konnte. Während für die Säure-Base Regulation wichtige Gene bei einer erhöhten Seewasser CO_2-Konzentration weder in Larven noch in Adulten verändert waren, konnte in den Larven eine erhöhte Genexpression einiger Gene der mitochondrialen Energiegewinnung unter erhöhtem Seewasser CO_2 Partialdruck nachgewiesen werden. Eine gesteigerte Genexpression dieser mitochondrialen Gene könnte auf eine mitochondriale Akklimation an einen erhöhten Seewasser Partialdruck hindeuten. Der Sauerstoffverbrauch der Larven und der adulten Tieren war jedoch unabhängig von der Seewasser CO_2-Konzentration. Der Einfluss eines erhöhten Seewasser CO_2 Partialdrucks auf den Säure-Base Haushalt und den Metabolismus beeinträchtigte nicht das Überleben der Larven oder adulten Tiere.

Daher wurden weiterführende Studien an frühen und möglicherweise sensitiveren Entwicklungsstadien durchgeführt. Der Einfluss einer erhöhten Seewasser CO_2-Konzentration auf die Entwicklung, das Wachstum und das Überleben des ersten Larvenstadiums (Zoea), die eine unterschiedliche Vorgeschichte bezüglich ihrer Embryonalphase hatten (hohe Seewasser CO_2-Konzentration vor und nach dem Schlupf vs.

normokapnische Seewasser CO_2-Konzentration vor dem Schlupf und eine erhöhte Seewasser CO_2-Konzentration nach dem Schlupf), wurde untersucht. Es zeigte sich ein geringer Einfluss von hohen CO_2-Konzentrationen auf den Sauerstoffverbrauch und das Wachstum, wenn die Larven diesen ausschließlich nach dem Schlupf ausgesetzt waren. Die Entwicklungszeit und das Überleben der Larven waren gänzlich unbeeinflusst. Im Gegensatz dazu zeigten Zoea Larven, die vor und nach dem Schlupf einer erhöhten CO_2-Konzentration ausgesetzt waren, eine außerordentlich hohe Mortalität und eine starke Verzögerung in ihrer Entwicklung, die vermutlich aus einer Verschiebung des larvalen Energiebudgets und des daraus resultierenden unzureichenden Wachstums resultierte. Die beobachte erhöhte Mortalität dieser vorexponierten Zoea Larven war nicht die Folge von Veränderungen des pHs und P_{CO_2} im exponierten Ei. Es ist anzunehmen, dass die Embryonen ebenfalls nicht durch eine geringere Sauerstoffversorgung durch die Weibchen beeinträchtigt waren. Tatsächlich, konnte ein verändertes Brutpflegeverhalten der Weibchen mit einem erhöhten Aufwand der Eiventilation bei einer erhöhten Seewasser CO_2-Konzentration festgestellt werden. Dies könnte weitreichende Folgen für das Energiebudget der Weibchen haben.

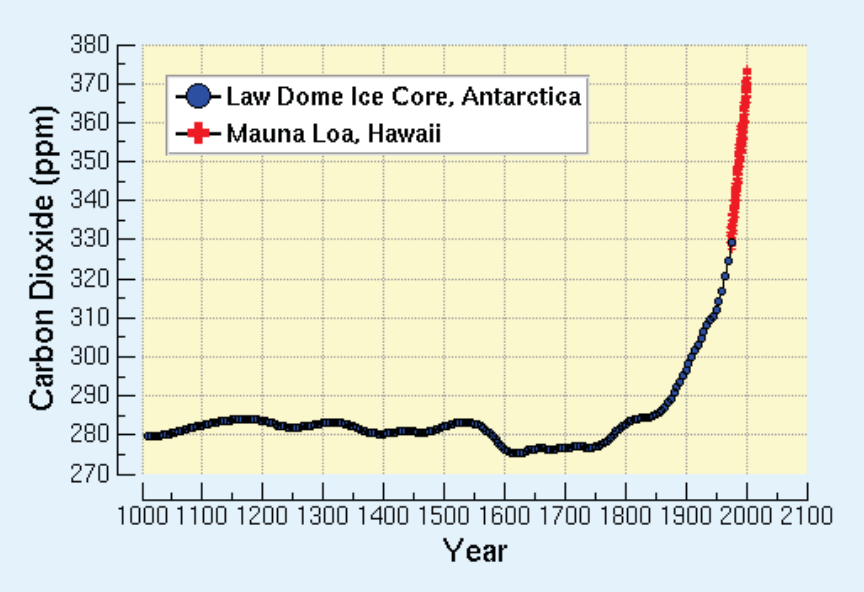
Um synergistische Effekte einer erhöhten Seewasser CO_2-Konzentration und erhöhter Temperaturen auf die larvalen Stadien zu untersuchen, wurden die Temperaturtoleranzfenster der Zoea und Megalopa Larven bestimmt, die verschiedenen CO_2-Konzentrationen ausgesetzt wurden. Eine erhöhte CO_2-Konzentration führte zu einer Limitierung der Temperaturtoleranz der Zoea Larven. Die Ergebnisse deuten darauf hin, dass eine erhöhte CO_2-Konzentration und erhöhte Temperatur synergistisch auf physiologische Mechanismen der Larven wirken könnten. Ein vergleichender molekularer Ansatz zeigte eine erhöhte transkriptomische Hitzeschockantwort verbunden mit einer erhöhten Genexpression von Hitzeschock Proteinen unter erhöhtem Seewasser CO_2 Partialdruck.

Die Ergebnisse der vorliegenden Arbeit deuten darauf hin, dass osmokonforme Krebse vergleichsweise toleranter gegenüber Ozeanversauerung sind. Dennoch konnte ein schwerwiegender Einfluss von erhöhten Seewasser CO_2-Konzentrationen auf einige frühe Lebensstadien von *H. araneus* nachgewiesen werden. Der Übergang vom Embryo zur frühen Larve könnte einen kritischen Engpass innerhalb des Lebenszyklus von *H. araneus* darstellen. Effekte, die sich von einem zum nächsten Lebensstadium übertragen und /oder CO_2 verursachte Störungen der Übergangsphasen zwischen einem und dem nächsten Stadium haben das Potenzial das Überleben von Arten maßgeblich zu gefährden.

1 Introduction

1.1 Ocean acidification and warming

The ongoing acidification and warming of the world's oceans due to anthropogenic CO_2 emissions into the atmosphere is receiving increasing public interest. CO_2 accumulates in the atmosphere and acts as a greenhouse gas. Anthropogenic burning of fossil fuels has already lead to an increase of atmospheric CO_2 from about 280 ppm, during pre-industrial times, to a value of 380 ppm in the year 2000 (Fig. 1.1, NOAA/ESRL, www.esrl.noaa.gov/gmd/ccgg/trends). This relates to a 36 % increase of atmospherical CO_2 in the last 200 years alone (Fig.1.1). There are, however, regional differences in the magnitudes of CO_2 increases and global distribution of atmospheric CO_2, with a higher content of atmospheric CO_2 in the marine boundary layer of the high northern latitudes (NOAA/ESRL, www.esrl.noaa.gov/gmd/ccgg/trends).



Fig, 1.1 Atmospheric CO_2 content during the last millennium determined from Antarctic ice cores and measured at Mauna Loa, Hawaii. Law Dome Ice Core data source: Etheridge et al. 1998. NOAA/ESRL, www.esrl.noaa.gov/gmd/ccgg/trends.

Based on different assumptions of anthropogenic CO_2 emissions, the Intergovernmental Panel on Climate Change presented different projections on future CO_2 rise (IPCC 2001, 2007). Projections are made assuming an ongoing increase in

atmospheric CO_2 (SRES pathways) or a stabilization of atmospheric P_{CO_2} (WRE scenarios) (Caldeira and Wickett 2005)(Fig. 1.2). A2 and B1 represent the highest and lowest CO_2 SRES emission scenario. The A2 scenario assumes large regional differences in technological progress, technological improvements and access to mineral resources. It predicts an increase of atmospheric P_{CO_2} to 970 ppm by the end of this century (Fig. 1.2). The story line behind the B1 scenario is a world with a globally coherent approach to sustainable development with a high level of environmental and social consciousness and therefore predicts a lower increase in atmospheric CO_2 to 650 ppm by the end of the century (Caldeira and Wickett 2005). WRE scenarios assume transition to different levels of stable atmospheric CO_2 concentrations by the year 2300 (Fig. 1.2), which still are considered to elicit changes in ocean chemistry. Further scenarios depending on the release of specified amounts of CO_2 to the atmosphere over the next several centuries project an increase in atmospheric CO_2 of up to 3000 ppm by the year 2300 (IPCC 2001).

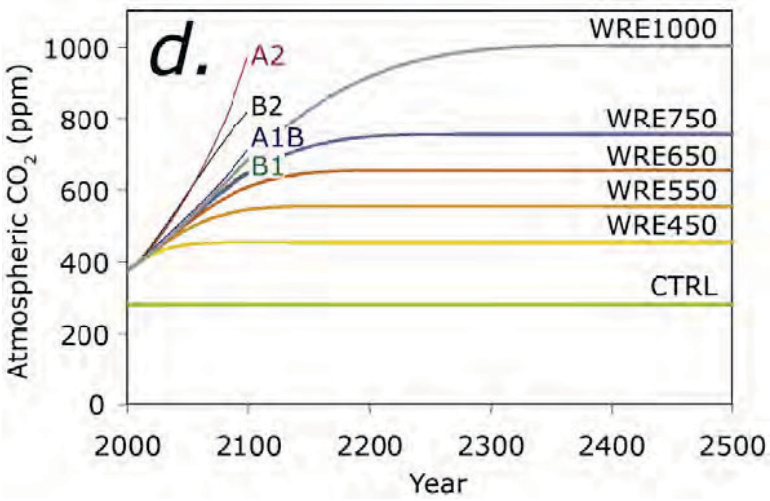


Fig. 1.2 Atmospheric CO_2 predicted for the SRES emission pathways and for the WRE stabilization scenarios with a stabilized final P_{CO_2} by the year 2300. Figure 1d from Caldeira and Wickett 2005.

Before the industrial revolution, less than half of anthropogenic CO_2 emissions remained in the atmosphere. The rest was taken up by equal amounts by the land biosphere and the ocean (Sabine et al. 2004). Without this oceanic uptake, present atmospheric CO_2 would be about 55 ppm higher than currently observed. Atmospheric CO_2 dissolves in seawater and forms carbonic acid (H_2CO_3), which dissociates into protons and bicarbonate (HCO_3) and further in protons and carbonate (CO_3^{2-}). The carbonate

species are formed according to the following equilibrium (Zeebe and Wolf-Gladrow 2001):

$$CO_2 + H_2O \overset{K_0}{\leftrightarrow} H_2CO_3 \overset{K_1}{\leftrightarrow} HCO_3^- + H^+ \overset{K_2}{\leftrightarrow} CO_3^{2-} + 2H^+$$

The chemical equilibrium is described by the equilibrium constants K_0, K_1 and K_2, which depend on salinity, temperature and pressure. The percentage of dissolved species CO_2, HCO_3^- and CO_3^{2-} is 0.5%, 86.5% and 13% respectively at a seawater pH of 8.1, a salinity of 35 and a temperature of 25°C (Zeebe and Wolf-Gladrow 2001).

Increasing atmospheric CO_2 leads to an increase in seawater pCO_2 and causes a decrease in seawater pH, due to the elevated release of protons during the dissolution of CO_2 in seawater. This effect is termed ocean acidification. Furthermore, elevated levels of protons react with carbonate ions resulting in formation of bicarbonate and decreasing carbonate concentrations. Today's changes of atmospheric CO_2 have already caused ocean pH to decline by more than 0.1 units below values characterizing pre-industrial times (Caldeira and Wickett 2003). Based on the SRES pathways, global surface pH levels are predicted to decrease between 0.3 and 0.5 pH units by the year 2100 (Caldeira and Wickett 2005). Global fossil fuel resources were estimated to be roughly 5000 Pg C (IPCC 2001). Burning of 5000 Pg C would result in an atmospheric CO_2 concentration of 2000 ppm until the year 2300 and cause pH to fall by 0.8 units below pre-industrial vales (Caldeira and Wickett 2005). Again, there are regional differences in the magnitude of acidification. Since colder seawater displays higher solubility for CO_2, it has been predicted that the largest pH changes of the 21st century will occur in the Arctic Ocean. A reduction of seawater pH by more than 0.3 pH units might occur by the end of the century (Fig. 1.3) (Cao and Caldeira 2008).

Ocean acidification goes hand in hand with ocean warming due to greenhouse effects. Another set of scenarios known as Representative Concentration Pathways describe the possible future evolution of atmospheric CO_2 (Moss et al. 2008). Depending on the scenario, sea surface temperatures are predicted to reach 1.5 to 8°C above pre-industrial values by the year 2300 (Meinshausen et al. 2011). During the last century temperature in the world´s oceans has already increased by a mean of 0.31°C in the 0 to 300 meter layer (Hegerl and Bindoff 2005). Atmospheric temperatures are predicted to rise a further 0.2°C per decade over the next two decades (IPCC 2007) and between 1.7°C and 2.5°C by the year 2100 (IPCC 2001), entailing further ocean warming.

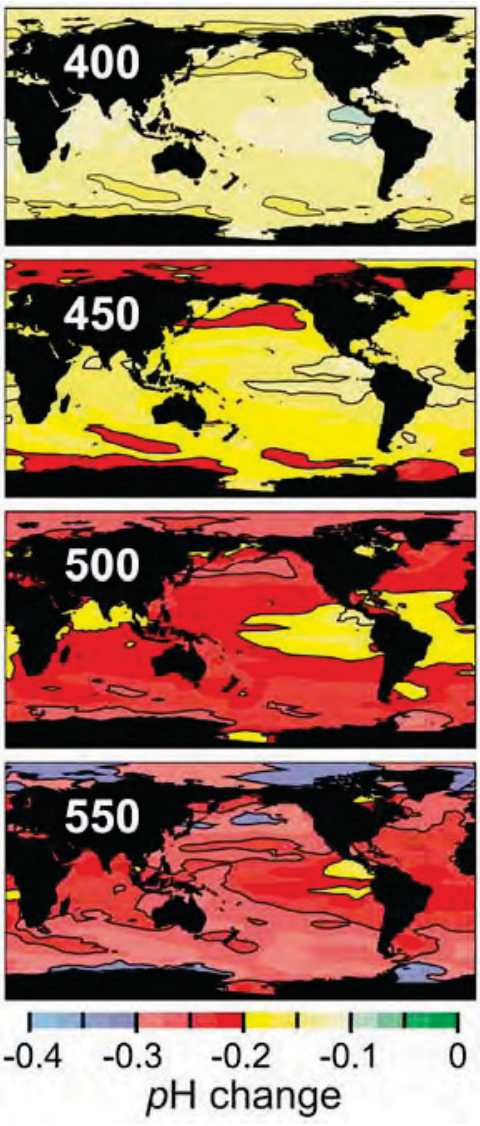


Fig.1.3 Ocean pH change. Changes in surface ocean pH relative to pre-industrial values for different atmospheric CO_2 stabilization levels. From Cao and Caldeira 2008.

1.2 Biological impacts of ocean acidification and warming

1.2.1 Physiological responses to elevated seawater P_{CO_2}

Various effects of elevated P_{CO_2} on the physiology of marine organisms have already been described. A crucial trait and a common feature of CO_2 tolerant species is the ability to compensate for pH disturbances of extracellular fluids during hypercapnic exposure (Melzner et al. 2009b) as the first line of defence. As the exchange of metabolic CO_2 between the extracellular fluid and the seawater is mainly achieved by means of diffusion, increasing seawater CO_2 levels lead to a smaller CO_2 gradient between the extracellular fluids and the surrounding seawater causing an increase of CO_2 in the haemolymph. Elevated haemolymph CO_2 levels involve an elevated release of protons, due

to the chemical equilibrium of carbonate species (see chapter 1.1), and a decrease in haemolymph pH. A low intra- and extracellular pH can have a depressing effect on protein synthesis (Langenbuch and Pörtner 2002) and aerobic metabolism (Barnhart and McMahon 1988, Reipschläger and Pörtner 1996) and can affect the binding of oxygen to the respiratory proteins (Weber and Hagerman 1981, Jokumsen et al. 1981). Thus, the buffering of protons is a crucial prerequisite to maintaining functional integrity and can be achieved via the CO_2-bicarbonate system itself, the non-bicarbonate buffering system and active ion transport across specialized epithelia, such as gills, renal or digestive tissue. The non-bicarbonate buffering system plays a central role in marine organisms (Melzner et al. 2009b). Non-bicarbonate buffers are proteins (e.g. respiratory proteins), amino acids or organic/inorganic phosphate groups (Somero 1981, Henry and Wheatly 1992). While the passive non-bicarbonate buffering system acts to buffer intra- and extracellular pH, only active ion-transport mechanisms can eliminate protons and restore pH. This involves the accumulation of bicarbonate (Truchot 1975, Cameron 1978, Claiborne and Heisler 1986). In marine crustaceans, gills are the main sites for the regulation of haemolymph acid-base balance via the exchange of acid/base equivalents with the seawater (Henry and Cameron 1983, Henry and Wheatly 1992).

Our understanding of acid-base regulation and the contribution of different ion transporters is far from being complete. The enzyme carbonic anhydrase (CA) is considered to be hugely important for ion regulation processes. Within the gill epithelia of crustaceans, metabolic CO_2 is hydrated by carbonic anhydrase into H^+ and HCO_3^-, which act as counterions for Na^+ and Cl^- transport processes (Henry and Cameron 1983). H^+ ions are extracted into the seawater via the passive Na^+/H^+ exchanger (NHE), which is driven by ion gradients established by the ATP consuming Na^+/K^+ATPase. HCO_3^- can be released into the plasma by means of basolateral Cl^-/HCO_3^- exchangers or Na^+/HCO_3^- co-transporters (Melzner et al. 2009b). As the Na^+/K^+ATPase is the main driving force for ion regulation and compensation of acid-base disturbances, it is widely used as an indicator for the overall capacity to deal with hypercapnic conditions (Deigweiher et al. 2008, Melzner et al. 2009a, Hu et al. 2011). Studies revealed a high inter-taxa and inter-species variation concerning Na^+/K^+ATPase activities. Na^+/K^+ATPase activities of hypercapnia tolerant marine taxa are roughly 1 order of magnitude higher than those of CO_2 sensitive taxa (Melzner et al. 2009b).

Some species within the order Decapoda order are highly tolerant towards ocean acidification due to high ion regulating capacities. The shore crab *Carcinus maenas* exhibits high Na^+/K^+ATPase capacities (Lucu and Flik 1999) and efficiently accumulates HCO_3 in its haemolymph during hypercapnic conditions of 6900 µatm (Truchot 1979). The decapod

crabs *Necora puber* and *Cancer magister* were able to recover their haemolymph pH over 24 h at a seawater P_{CO_2} of 8000 µatm (Pane and Barry 2007, Spicer et al. 2007). However, ion regulation capacities seem to be related to the variances in chemical environment experienced by a species during daily life. Whilst the shallow-living Dungeness crab, *C. magister,* was able to fully recover the haemolymph pH by accumulating HCO_3^- from the medium, no compensation for acid-base haemolymph disturbances could be found in the deep-sea Tanner crab *Chionoecetes tanneri* exposed to elevated seawater P_{CO_2} (Pane and Barry 2007).

On the one hand, extra- and intracellular ion regulation and the establishment of ion gradients is energy consuming with a transporter dependent stoichiometry of 0.17 – 0.5 ATP per proton (Pörtner et al. 2000) and will likely affect the organisms' energy budget. In marine teleost fish, gill Na^+/K^+ATPase activity and whole animal metabolic rate showed a positive correlation (Gibbs and Somero 1990). A drop of extracellular pH during hypercapnic exposure might, if compensated for by enhanced acid-base regulation processes, thus lead to a rise in energy demands (Thomsen and Melzner 2010, Stumpp et al. 2011a). On the other hand, uncompensated extra- and/or intracellular acid-base disturbances can elicit metabolic depression and lower whole animal oxygen consumption (Reipschläger and Pörtner 1996, Michaelidis et al. 2005).

Enhanced energetic costs for acid-base regulation during hypercapnic exposure therefore have the potential to shape an organisms' energy budget and allocation as they diminish the energy available for other crucial components of the energy budget like growth (Stumpp et al. 2011 a,b). In the tropical prawn *Macrobrachium rosenbergii*, rates of protein biosynthesis, a determinant of growth, were highest when the minimal amount of energy was consumed by ion regulation processes (Intanai et al. 2009). However, metabolic depression was also accompanied by reduced protein biosynthesis under conditions of severe acidosis in isolated liver cells of Antarctic fish (Langenbuch and Pörtner 2003). Obviously, lower oxygen uptake and reduced metabolic rates, resulting from hypercapnic exposure, will involve less energy available for growth.

1.2.2 CO_2 sensitivities of early life history stages

Evidence is growing that high seawater P_{CO_2} has profound effects on the development of early life stages in marine ectotherms (reviewed by Kurihara 2008, Fig. 1.6). Early ontogenetic stages (fertilization, cleavage, embryos) seem to be especially vulnerable. Embryos of the Sydney rock oyster, *Saccostrea glomerata,* that were exposed to high CO_2 and high temperature for fertilization and embryonic development had fewer

D-veligers with greater percentage of abnormalities as well as reduced size compared to embryos that were exposed to high CO_2 and high temperature for embryonic development only (Parker et al. 2009). Also in the sea urchin, *Heliocidaris erythrogramma,* sperm swimming speed and percent sperm motility as well as developmental success of embryos was reduced at low pH of 7.7 (Havenhand et al. 2008). Most of the studies regarding the effects of elevated seawater CO_2 concentrations on early ontogenetic stages of crustaceans focused on copepods and amphipods (Kurihara and Ishimatsu 2008, Zhang et al. 2011, Egilsdottir et al. 2009). Neither the developmental time nor number of hatchlings were affected by high seawater P_{CO_2} of 1900 µatm in the amphipod *Echinogammarus marinus* (Egilsdottir et al. 2009). Similarly in the copepod *Acartia tsuensis*, egg production, hatching rate and egg survival at a seawater P_{CO_2} of 2000 µatm had no effect (Kurihara and Ishimatsu 2008). A negative effect on hatching rate and nauplius mortality could be found at extremely high seawater P_{CO_2} values of 5000 and 10000 µatm in the copepod *Acartia erythraea* (Kurihara et al. 2004). However, there was high inter-species variation concerning CO_2 effects on early life developmental stages of copepods. Two out of four different copepod species showed reduced egg hatching success and egg survival at elevated seawater P_{CO_2} (Zhang et al. 2011).

The embryonic development of e.g. fish, crustaceans, cephalopods and gastropods within a protective egg capsule might be especially challenging at enhanced seawater CO_2 levels. Respiratory gases (O_2 and CO_2) of embryos have to pass the egg wall by means of diffusion gradients. Elevated seawater P_{CO_2} might result in an increase in egg fluid P_{CO_2} to maintain a sufficient CO_2 gradient from the egg to the seawater. Previous work has demonstrated that the P_{O_2} and pH decreased whilst P_{CO_2} increased in the perivitelline fluid (PVF) of embryos of the cuttlefish *Sepia officinalis* with increasing embryo wet mass (Gutowska and Melzner 2009). The already high PVF P_{CO_2} had almost quadrupled and reached values of 7800 µatm, when *S. officinalis* eggs were reared at high seawater P_{CO_2} of 3700 µatm (Hu et al. 2011). Only very limited data exists on the abiotic conditions within the egg capsule of other marine invertebrates.

The eggs of almost all crustaceans are attached to the female's pleopods and are subject to maternal care. Thus, embryos are not only exposed to the same environmental conditions as the ovigerous females but their well-being and finally, recruitment also depend on the performance capacity of the female to facilitate gas exchange of the egg masses (Fernández et al. 2000). Ovigerous females have been proposed to be potentially sensitive to environmental stress (Pörtner and Farrell 2008) due to higher costs of parental care (Fernández et al. 2000) and eggs may be affected in two ways: less oxygen supply by the females due to CO_2 stress and/or higher costs for extracellular acid-base

regulation and, second, high CO_2 sensitivity for the eggs. As mentioned above, acid-base regulation can be achieved via active ion transport across specialized epithelia. The ontogeny of ion regulation of crustacean embryos seem to be linked to variability in salinity of the female's/adult's habitat (Charmantier and Charmantier-Daures 2001). The more variable the salinity is in the adult habitat, the earlier osmoregulation is established in development. Thus, embryos and ovigerous females of subtidal or deep-sea species, adapted to stable environmental conditions, might be especially vulnerable to ocean acidification.

Exposure to elevated P_{CO_2} also affects larval stages of marine organisms. Hypercapnic exposure resulted in severe to lethal tissue damage in Atlantic cod larvae (Frommel et al. 2012). Tissue damage was found in the liver, pancreas, kidney, eye and the gut with the degree of damage increasing with CO_2 concentration. Larvae of another commercial important fish species, the Atlantic herring, exhibited lower RNA concentrations at hatching at high CO_2 (Franke and Clemmesen 2011). Ocean acidification also impaired the olfactory discrimination and homing ability of coral fish larvae (Munday et al. 2009b). Calcifying larvae of echinoderms seem to be especially sensitive even to slightly higher seawater CO_2 concentrations. Acidification by 0.2 units induced 100 % larval mortality in the Atlantic keystone species *Ophiothrix fragilis* and resulted in decreased larval growth as well as abnormal development (Dupont et al. 2008). Reduced growth and delayed development was also elicited by elevated seawater P_{CO_2} in larvae of the sea urchin *Strongylocentrotus purpuratus* (Stumpp et al. 2011a,b). Results from these and other studies suggest that high seawater CO_2 concentrations mainly affect larval skeleton and shell synthesis of marine calcifiers resulting in reduced growth and delayed developmental timing (review Kurihara 2008).

This pattern also holds true for crustacean larvae. Larvae of the spider crab *Hyas araneus* and the lobster *Homarus gammarus* showed altered growth and calcification rates at high seawater CO_2 concentrations (Arnold et al. 2009, Walther et al. 2010, Walther et al. 2011). Growth rates were similarly reduced in the early life stages of the barnacle *Semibalanus balanoides* reared at 1000 µatm CO_2 (Findlay et al. 2010). Zoea I and zoea II larvae of *H. araneus* exhibited delayed development at elevated seawater P_{CO_2} (Walther et al. 2010). Whilst early life stages of marine calcifiers are more sensitive to high seawater P_{CO_2} levels than adults (review Kurihara 2008), the physiological background of their increased sensitivities has not yet been comprehensively explored.

1.2.3 Synergy between CO_2 and temperature

The on-going increase of seawater PCO_2 is occurring in synergy with other environmental changes (increase in ocean temperature, expansion of hypoxic zones). The cumulative or interactive impact of multiple stressors might be even more severe for marine organisms than the stressors individually. Marine organisms are sensitive to changes in seawater PCO_2 and temperature with effects significantly increased when both stressors acted in synergy (Walther et al. 2009, Pansch et al. 2012, Zittier et al. 2012). A pattern is evolving in which marine calcifiers show higher ability to tolerate even severe hypercapnic stress when being kept in their optimal thermal window range (Rodolfo-Metalpa et al. 2011, Pansch et al. 2012).

According to the concept of oxygen- and capacity-dependent thermal tolerance, the optimal thermal window in aquatic ectotherms is characterized by the maximum scope for aerobic metabolism and aerobic energy is able to support all physiological functions and animal performances (Pörtner 2002). The setting of low and high temperature ranges results from temperature dependent trade-offs from the molecular to the systemic level. At temperature extremes the limited capacities of ventilatory and circulatory systems cause a mismatch between oxygen demand and oxygen supply leading to reduced aerobic scope and, as a consequence, thermal tolerance (Frederich and Pörtner 2000). A distinction is made between a narrow, ecologically relevant thermal tolerance window limited by the pejus temperatures (T_p) and a wider thermal window limited by critical temperatures (T_c) that defines individual physiological tolerance. Between the pejus temperatures, the aerobic scope of an organism is maximized by oxygen supply through ventilation and circulation; heart beat and ventilation are increasing with temperature to compensate for the rise in oxygen demand in the warmth. (Frederich and Pörtner 2000, Pörtner 2001, Wittmann et al. 2008, Storch et al. 2011). Beyond the T_p, limited capacities of ventilation and circulation lead to a progressive mismatch between oxygen demand for maintenance and oxygen supply and a decrease of haemolymph oxygen partial pressure (Walther et al. 2009). Upon further cooling or warming, hypoxia and, finally, anaerobic metabolism occurs, where the organism reaches its critical temperature. Survival beyond T_c is time-limited and generally accompanied by an increase in anaerobic end products (Pörtner and Zielinski 1998, Frederich and Pörtner 2000). At the upper end of the thermal tolerance window, further warming might elicit loss of protein function, the heat shock response and oxidative stress (denaturation temperature T_d) (Pörtner 2001).

The optimal performance of the animal is the basis for a species' fitness including growth and survival. There is already evidence that elevated seawater PCO_2 reduces animal performance, especially at the limits of the thermal window (Walther et al. 2009, Munday

et al. 2009a). The mechanistic interactions of elevated seawater P_{CO_2} and temperature extremes have been proposed to narrow the thermal tolerance window of an organism exposed to high CO_2 levels (Pörtner and Farrell 2008, Pörtner 2008). Depending on elevated seawater CO_2 concentrations, upper thermal tolerance limits have been observed to be lowered by several °C in adult crustaceans (Metzger et al. 2007, Walther et al. 2009).

To understand the synergistic effects of increasing seawater P_{CO_2} and temperature on a population level, it is important to include the most vulnerable life cycle stages. Early developmental stages are suggested to be most sensitive to environmental hypercapnia (Kurihara 2008) and to possess narrow thermal windows (Pörtner and Farrell 2008, Pörtner 2010). They might, thus, be a bottleneck for successful survival and viability of a species in a warm and high CO_2 ocean. So far, there is limited data available on the thermal tolerance of larval stages exposed to elevated seawater P_{CO_2}.

The limitation in thermal tolerance during hypercapnic exposure might be due to different underlying mechanisms and mechanistic interactions (Pörtner 2010):

1) As described above, CO_2 sensitivities of different marine taxa seem to be highly dependent on their capacities to regulate blood acid-base disturbances at elevated seawater P_{CO_2} (Melzner et al. 2009b). The **capacity to regulate acid-base disturbances** might become limited when organisms are exposed to temperature extremes. As elevated seawater CO_2 and temperature concomitantly affect the acid-base status, strong acid-base disturbances leading to reduced protein function may be responsible for a lower temperature tolerance in organisms exposed to elevated CO_2.

2) Individual mechanisms affected by CO_2, hypoxia and temperature result in metabolic reduction and functional flexibility extending the period an organism is able to passively withstand environmental extremes (Pörtner et al. 2005). Systemic hypercapnia causes metabolic depression by shifting the acid-base status and increasing gas partial pressure gradients (Pörtner et al. 1998). While **metabolic depression** under elevated CO_2 will support extended survival at thermal extremes, it will also reduce the organisms' capacity to increase its rate of aerobic energy turnover within its optimal temperature range (Pörtner et al. 2005). Thus transgression to pejus conditions occurs more rapidly, resulting in limited performance and growth functions.

3) Elevated seawater P_{CO_2} affects **the heat shock response** of larvae and adult marine ectotherms (Todgham and Hofmann 2009, O'Donnell et al. 2009, Chapman et al. 2011). Responses varied from reduced capacity of cellular stress responses, measured by gene

expression of heat shock proteins (Todgham and Hofmann 2009, O'Donnell et al. 2009), to up-regulation of heat shock proteins at low pH (Chapman et al. 2011).

1.3 *Hyas araneus*: a model organism

1.3.1 Osmoconforming crustaceans

Marine crustaceans make up the majority of the 68 000 species described to date (Martin and Davis 2006). They have occupied a variety of habitats and ecological niches. This already emphasizes the outstanding status of crustaceans for climate change research as pointed out by Whiteley (2011). Marine crustaceans experience different degrees of environmental variability during their daily life. While deep sea and high latitude crustaceans live in stable environments, intertidal and estuary species are exposed to rapidly changing fluctuations in physical factors (Whiteley 2011). Consequently, crustaceans show a variety of physiological responses to changes in abiotic conditions. While some species regulate against external salinity changes, others simply conform. As acid-base regulatory mechanisms are crucial to cope changes in seawater CO_2 concentration, studies on crustaceans might help to correlate environmental variability with capacities for CO_2 tolerance (Whiteley 2011).

To date, most studies have dealt with the effect of elevated seawater P_{CO_2} on osmoregulating crustacean species. However, poor osmoregulators or osmoconformers might be especially sensitive towards ocean acidification due to limited compensatory capacities to regulate acid-base disturbances. The ability to deal with ocean acidification effects are even lower in slow-moving inactive species with low haemolymph protein levels and accordingly lower buffering capacities (Whiteley 2011). Deep sea and high latitude crustaceans with low metabolic rates might be more vulnerable due to limited metabolic capacities to cope with environmental change (Pane and Barry 2007, Whiteley 2011).

Hyas araneus is an osmoconforming and calcifying decapod crab (Fig. 1.4). It is a continental shelf species, which inhabits rocky, sandy and muddy bottoms. Adult individuals can be found at depths of 1-360 m, most commonly at <50 m (Christiansen 1969). *H. araneus* is an ideal model organism to study the effects of elevated seawater P_{CO_2} as it represents an osmoconforming, slow-moving inactive member of the ecologically important marine group of decapod crustaceans, which might be especially vulnerable to ocean acidification.

Fig. 1.4 Adult male of the spider crab *Hyas araneus* (Picture M. Schiffer).

1.3.2 Distribution

Hyas araneus has a wide distribution range from the English Channel and temperate southern North Sea to arctic waters (Christiansen 1969) (Fig. 1.5). Different populations of *H. araneus* experience a variety of environmental temperatures. The northernmost population in the Arctic Kongsfjord (Spitzbergen) exists in a small temperature range from 0-6°C (Svendsen et al. 2002), while the southernmost populations experience a broader temperature range between 3°C and 18°C (Wiltshire and Manly 2004).

Fig. 1.5 *Hyas araneus* distribution from the English Channel and temperate southern North Sea to subarctic waters. Modified from Hugo Ahlenius, UNEP/GRID-Arendal.

H. araneus populations within the latitudinal gradient might have adapted to their habitats as they encounter a wide variety of local conditions and individuals from different populations might have become differentiated in terms of e.g. their thermal sensitivities. Population-dependent levels of sensitivities to climate change can have implications for the future spatial distribution of species and can shape its biogeography. Increasing temperature in the North Sea during the last 40 years has already led to a drastic decrease in the abundance of *H. araneus* around Helgoland (Walther et al. 2010) suggesting similar effects on the southernmost populations.

Due to its wide distribution range along a latitudinal gradient, *H. araneus* is an ideal model organism to study the effects of increasing seawater PCO_2 and temperature in populations adapted to different environmental regimes. Comparisons between *H. araneus* populations have already revealed different CO_2 sensitivities of larvae from a temperate and an arctic population. While elevated seawater PCO_2 negatively affected the growth and development of temperate larvae, arctic larvae respond more sensitively to thermal stress than to enhanced CO_2 levels (Walther et al. 2010). Further studies will improve predictions of the relationship between environmental variability and species capacity to tolerate ocean acidification and warming.

1.3.3 Reproduction and larval development

Development and growth of *Hyas araneus* comprises several embryonic, larval and juvenile stages (Anger 1984, Petersen 1995) (Fig. 1.6). Ovigerous females carry the egg masses for approximately 2 years (Petersen 1995). The embryonic development of *H. araneus* was studied in a temperate population and was divided into four phases (Petersen 1995): Phase I is characterized by cleavage and lasts for one month. Phase II is characterized by gastrulation and takes (dependent on temperature) between two and eleven months. During the subsequent eight-month phase III, eye-pigment, heart beat, appendages and pigmentation become visible. Phase IV is the final phase and the dark and now small yolk-filled area in the eggs divides into two separate parts. Embryos begin to move inside the eggs and will hatch between one and two months later. Phase II is considered a diapause and determines the duration of embryonic development (one or two year embryonic development) (Petersen 1995). It might be an adaptation to more northern habitats with highly seasonal occurrence of planktonic food for the larvae (Petersen 1995).

Developmental duration of the larval stages of *H. araneus* varies highly with temperature and population (Anger 1983, Walther et al. 2010). The two zoea stages are pelagic and use their maxillipeds to actively swim in the upper water column. They have a characteristic spherical carapace shape and show long dorsal and lateral carapace spines (Anger 2001). The general appearance of the second zoea stage is similar to the first zoea beside the greater size and stalked eyes. The appearance of megalopa larvae marks the transition from a pelagic to a benthic mode of life. The megalopa larvae differs morphologically from the zoea larvae. It is similar to the benthic juvenile crab and has well developed walking legs (Anger 2001). The megalopa can also swim with natatory pleopods, which are reduced in juveniles. Cuticular calcification increases during the megalopa stage. It selects a suitable habitat before moulting into the first juvenile (crab I). Subsequently, *H. araneus* grows and develops through a high number of juvenile stages until finally moulting into a mature adult.

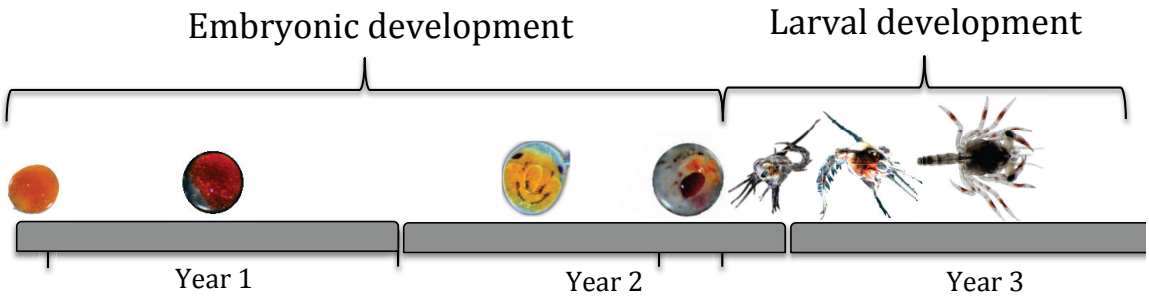


Fig.1.6 Embryonic and larval development of *Hyas araneus* redrawn from Anger (1984) and Petersen (1995).

H. araneus is a model organism to study the effects of elevated seawater PCO_2 on successive life-history stages. Broad knowledge exists on its larval development depending on several abiotic conditions (Anger 2001). It has few life stages, which are clearly distinguishable and occupy habitats with different environmental variability and might thus be differentially affected by ocean acidification and warming.

1.4 Approaches and questions

The aim of the present study was to apply an integrative approach to link cellular to whole organism responses to ocean acidification and warming in different life stages of the osmoconforming spider crab *Hyas araneus*. Although osmoconforming crustaceans have been proposed to be especially vulnerable to ocean acidification, the acid-base regulatory capacities of an osmoconforming crustacean have not been studied in

combination with the examination of growth and metabolism during long-term exposure to elevated seawater P_{CO_2}. Recent findings suggest the megalopa stage as a potential bottleneck within the life cycle of *H. araneus* as growth and calcification were compromised at elevated seawater P_{CO_2} (Walther et al. 2010, 2011). In the present thesis, multiple life history stages (embryos, zoea larvae, ovigerous females and adult males) were considered to evaluate other potential bottlenecks within the life cycle of *H. araneus* as they can drive the whole species response to ocean acidification and warming.

More specifically the following questions were addressed:

1) *Can different life cycle stages of the osmoconforming Hyas araneus regulate hypercapnia-induced acid-base disturbances? Does a disruption or regulation of the acid-base status compromise metabolic regulation in Hyas araneus?*

In order to achieve a comprehensive picture of the capacity to regulate the extracellular acid-base status during hypercapnic exposure, unpublished data obtained during the thesis were combined with results from publications I and III. On the whole-animal level, extracellular pH was determined after ten weeks and fourteen weeks of exposure in adult male and female *H. araneus*, respectively. Using the homogenization technique described by Pörtner et al. (1990), egg pH was examined in eggs of *H. araneus*. On the molecular level, the gene expression of ion transporters and enzymes involved in acid-base homeostasis were measured in eggs, larvae and adult crabs.

At the whole-animal level, oxygen consumption rates of adult males and larvae of *H. araneus* were measured after long-term incubations to elevated seawater CO_2 to test the assumption that a disruption or regulation of the acid-base status compromises the metabolism. On the molecular level, the gene expression of genes involved in mitochondrial energy metabolism was determined in larval stages of *H. araneus*.

2) *Are early developmental stages (embryos, larvae) the most CO_2 sensitive life cycle stages of Hyas araneus?*

The impact of high seawater CO_2 on development, growth and survival of zoea I larvae with different pre-hatch history was examined to test the assumption that early developmental stages are a potential bottleneck within the life cycle of marine calcifiers exposed to hypercapnic conditions. Zoea I larvae of *H. araneus* were exposed to high CO_2 during pre-and post-hatching phase and to normocapnic CO_2 during pre-hatching phase

and high CO_2 during post-hatching phase, respectively (publication I and II). Larval survival and developmental duration was examined. Furthermore, changes in the dry weight increment and oxygen consumption rate were determined during the time course of the first larval stage. The key question of this suite of experiments was whether effects of elevated seawater P_{CO_2} during the pre-hatching phase can carry-over to the first larval stage.

3) *Is the thermal tolerance of Hyas araneus larvae limited under elevated seawater P_{CO_2} due to synergistic effects of hypercapnia and temperature on metabolism, acid-base regulation and/or heat shock response?*

Exposure to elevated seawater CO_2 narrowed the thermal window of adult *H. araneus* (Walther et al. 2009). In order to address synergistic effects of elevated seawater CO_2 and temperature on larvae of *H. araneus*, the temperature tolerance window of zoea and megalopa larvae exposed to different seawater P_{CO_2} was determined. On the whole-animal level oxygen consumption, heart rate and activity (maxilliped beat rate) were examined during acute warming. On the molecular level, the expression of genes involved in acid-base regulation, mitochondrial energy metabolism and the cellular stress response was measured in larvae exposed to different seawater CO_2 levels and an acute heat shock. The key question of these experiments was whether the above-described mechanistic interactions regarding acid-base disturbances, metabolic depression and the heat shock response are involved in limiting thermal tolerance during hypercapnic exposure.

2 Material and methods

2.1 Animals and maintenance of males and ovigerous females

Eight experiments were conducted with adults and larvae of two populations (Arctic: Kongsfjord; Temperate: Gullmarsfjord) of the spider crab *Hyas araneus* (Tab. 2.1).

Males and females of *H. araneus* used for experiments were transferred to the Alfred Wegener Institute (AWI) in Bremerhaven and maintained in flow-through aquaria at 32 psu and a constant dark: light cycle (12 h: 12 h). During this period, seawater was aerated with ambient air and animals were fed *ad libitum* with mussels (*Cerastoderma edule* and *Mytilus edulis*). For experiments 1, 2 and 3, male and ovigerous females of Arctic *H. araneus* were collected in the Kongsfjord (Ny Alesund, Svalbard 78°55 N, 11°56 E) by scientific divers during May 2009. Animals were maintained at the AWI in flow-through aquaria at 4°C. For experiments 4 and 5, ovigerous females were collected in the Kongsfjord by scientific divers during spring 2010. Animals were maintained at 4°C during summer and at 0°C during early winter to avoid early hatching. Male and ovigerous females of *H. araneus*, used in experiments 6, 7 and 8, were collected by local fishermen in the Gullmarsfjord (Sweden, 58°N) in September 2010. Animals were maintained in flow-through aquaria at 10°C at the AWI.

Tab. 2.1 Overview of population, life stage and experimental parameters used for the present thesis.

Population	Life stage	Long-term incubation				Measured parameters	Publication
		No	Duration (days)	Seawater CO_2 concentration (µatm)	Temp (°C)		
Kongsfjord	Adult male	1	10 weeks	440 990 1580 1880	5	Extracellular pH and CCO_2, haemocyanin oxygen binding, gene expression	Unpublished data
Kongsfjord	Adult male	2	10 weeks	370 940 2020 2900	10	Extracellular pH and CCO_2	Unpublished data
Kongsfjord	Zoea I larvae	3	7 weeks	490 1030 2340	6	Survival, developmental duration, metabolic rate, dry weight, C and N content	Publication 1
Kongsfjord	Eggs, zoea larvae	4	17 weeks	350 3100	4	Survival, developmental duration, metabolic rate, dry weight, feeding rate, survival under starvation, heart beat and maxilliped beat rate	Publication 2
Kongsfjord	Ovigerous females, eggs	5	14 weeks	450 2400	4	Egg metabolic rate, female brooding behaviour, egg pH and CCO_2	Unpublished data
Gullmarsfjord	Adult male	6	10 weeks	400 950 2070 2400	10	Extracellular pH and CCO_2, metabolic rate, carapace structure	Unpublished data
Gullmarsfjord	Adult male	7	10 weeks	380 920 1860 2490	16	Extracellular pH and CCO_2, metabolic rate	Unpublished data
Gullmarsfjord	Zoea and megalopa larvae	8	10 weeks	430 3390	10	Metabolic rate, heart beat and maxilliped beat rate, gene expression	Publication 3

2.2 Experimental set-up

2.2.1 Adults

Long-term experiments on male adults of *Hyas araneus* from both populations were conducted at the Alfred Wegener Institute in Bremerhaven. Experiments were set-up in a climate chamber to keep the temperature constant. Animals were exposed to four different CO_2 concentrations and two different temperatures, respectively, for 10 weeks (see Tab. 2.1). For each treatment, 4-9 animals were individually placed in 2 l wide-mouth container (Kautex, Bonn, Germany) or 2 l aquaria.

The 2 l wide-mouth container/aquaria were placed within a recirculating CO_2 incubation systems (volume 1 m^3 seawater each) and gravity fed by a header tank (Fig. 2.1). The flow rate was adjusted to 200 ml min^{-1} for each container. A seawater storage tank was used for thermal equilibration and aeration with pressurized air with a defined air / CO_2 mixture using an automatic mass flow controller (HTK 6 channel, HTK Hamburg GmbH, Germany). Seawater was pumped from the storage tank to the header tank. Water of experimental containers was recollected, transferred to a collection tank and pumped back to the storage tank at a flow rate of 20 l min^{-1}.

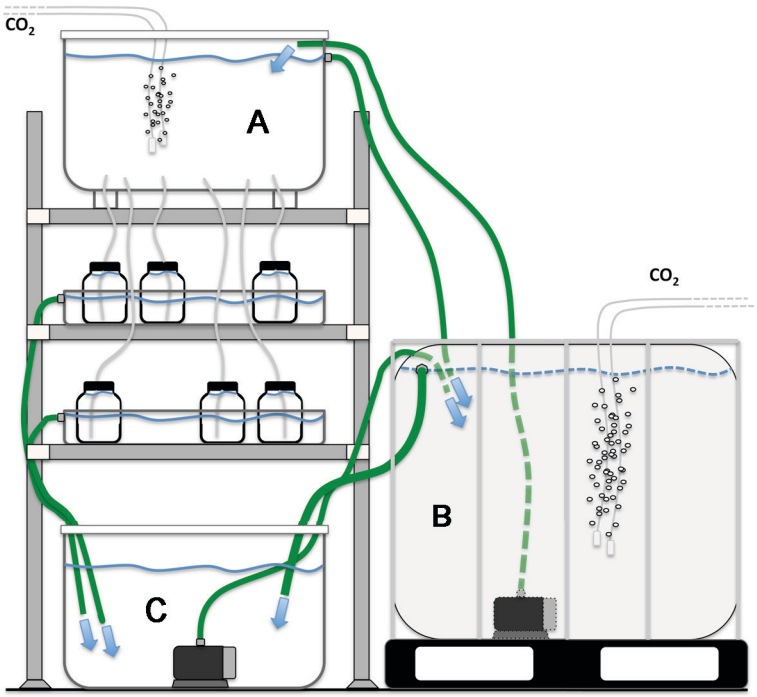


Fig. 2.1 Experimental set-up used for incubations of male and female *Hyas araneus*. Header tank (A), storage tank (B) and collection tank (C). The arrows indicate the direction of water flow (Drawing by Lars Harms).

The same experimental set-up was used in experiment 6 for a 2-week incubation of ovigerous females. Experiment 8 was conducted at the aquaria facilities of the Alfred Wegener Institute. Ovigerous and non-ovigerous females (n = 16-19) were maintained in flow-through aquaria at 4°C at two different seawater P_{CO_2} for 14 weeks (Tab. 2.1). Seawater CO_2 manipulation was achieved by injecting the aquaria with a defined air/CO_2 mixture using a mass flow controller (HTK 6 channel, HTK Hamburg GmbH, Germany). The flow rate was reduced to 500 ml/min in the high CO_2 treatment to guarantee steady state seawater acidification.

2.2.2 Larvae

Within the next section, I will summarize the larval collection and maintenance for all experiments (for details see material and methods publication I, II and III).

Newly hatched larvae of different females were pooled and transferred into 0.5 l culture vessels at a density of 30 individuals per vessel for the zoea larvae. The density was reduced to 15 larvae for the larger megalopa stage. All experiments were conducted with larvae that had hatched within 24 h. Larvae were reared in closed culture vessels filled with seawater of different CO_2 concentrations at a constant temperature. Zoea I that moulted into the zoea II stage or zoea II that moulted into the megalopa stage at the same day were pooled together in a new culture vessel. Seawater was provided from reservoir tanks (60 l) injected with a defined air/CO_2 mixture using a mass flow controller (HTK 6 channel, HTK Hamburg GmbH, Germany). Water of culture vessels was changed daily and dead larvae and moults were removed. Larvae were fed daily *ad libitum* with freshly hatched *Artemia* sp. (Sanders Brine Shrimp Company, Ogden, Utah, USA).

2.3 Whole animal performance

2.3.1 Larval survival and developmental time

In order to investigate the effect of elevated seawater CO_2 on the recruitment success of *Hyas araneus*, survival and developmental time were determined in larvae with different pre-hatch history (high CO_2 during pre-and post-hatching phase vs. normocapnic CO_2 during the pre-hatching phase and high CO_2 during the post-hatching phase).

A defined number of culture vessels per CO_2 concentration (3-11) were used to investigate the effect of elevated CO_2 on the mortality and duration of larval development. Mortality and moulting was recorded on a daily basis until all larvae were either dead or moulted into the next stage. Daily mortality and/or total mortality of larvae were

calculated for each vessel. For daily records of larval mortality, the percentual fraction given refers to the number of larvae remaining alive after each day. Culture vessels were used as (3-11) replicates and total mortality was determined as the mean of replicates. Mean developmental time for larvae of corresponding seawater CO_2 concentrations was determined as the mean of replicates or as median developmental time (see publication II for details).

2.3.2 Larval growth

2.3.2.1 Fitness proxies

Dry weight and elemental composition can be used as fitness proxies for larval crustaceans. Larvae were removed from seawater and briefly rinsed with deionized water. Excessive water was removed using a paper towel. Subsequently, larvae were stored at -20°C in pre-weighed tin cartridges for analyses. Individual larvae within the tin cartridges were freeze-dried and weighed on a high precision balance (Mettler Toledo AG, Greifensee, CH-8606, CH). After determination of dry weight, C and N content of larvae were measured in a CN analyzer (Euro EA-CN analyzer), using acetanilide as standard.

2.3.2.2 Feeding rates

Larval feeding behaviour was examined to compare the observed growth rates with the feeding rates (publication II). Larvae were held individually in closed vials containing 10 ml of seawater. One day prior to experiments vials were placed at the respective temperatures in a temperature-controlled table providing a stable temperature gradient (custom-made by AWI workshop). Larvae were starved for two days at rearing temperature and subsequently transferred to experimental temperatures (see publication II) for one day prior to feeding experiments. On day four, *Artemia* sp. (Sanders Brine Shrimp Company, Ogden, Utah, USA) were added at a density of 10 animals ml^{-1} per vial. After 24 h, the larvae were carefully removed from the incubation vial to avoid the withdrawal of any *Artemia*. The remaining *Artemia* were counted under a microscope.

2.3.2.3 Survival under starvation

Larvae were transferred individually into closed vials containing 10 ml seawater. One day prior to the experiments, vials were exposed to experimental temperatures (see publication III) in a temperature-controlled table. Seawater was provided from reservoir tanks that were injected with a defined air/CO_2 mixture. Water in the experimental vials

was changed daily and checked for dead larvae. Larvae were considered dead when no heart beat could be detected under a microscope.

2.3.3 Metabolic rates

Standard metabolic rates were measured in larvae and adults of *Hyas araneus* to examine energetic demands of different life stages at different seawater CO_2 concentrations and to correlate them to the observed growth rates and capacities of acid-base regulation.

2.3.3.1 Adults

Standard metabolic rates of adult *Hyas araneus* were measured via intermitted-flow respirometry. Animals were starved for 24 hours and subsequently transferred to a transparent, cylindric respirometer (1.6-2.8 l water volume) for 24 hours. The respiration chamber was placed within the recirculating CO_2 incubation systems (described in 2.2.1) to maintain temperature and CO_2 concentration. A constant water flow was generated using an aquarium pump (Eheim compact 300) and gas tight tubing. Applied flow rate ensured 100 % oxygen saturation within the chamber. During intermittent flow, water exchange between system and chamber was stopped for 15 or 30 minutes in intervals of 15 or 30 minutes and seawater circulated in the chamber without water influx. Oxygen depletion (max. 20 %) was measured using a fiber-optic oxygen sensing system (Oxy-4 Micro, PreSens GmbH, Regensburg, Germany) and needle type optodes, embedded into the recirculated loop. Oxygen consumption rates were calculated from declines in chamber oxygen partial pressure.

2.3.3.2 Larvae and eggs

Two different set-ups were used to measure oxygen consumption rates of larvae and eggs. For experiment 5, Hamilton high precision microliter syringes (500 µl, Hamilton-Bonaduz, Switzerland) were used as closed respiratory chambers. For experiments 6, 7 and 8, oxygen consumption rates of eggs and larvae were measured in closed, double-walled respiration chambers (OXY041 A, Collotec Meßtechnik GmbH, Niddatal, Germany) perfused by thermostatted water to maintain control temperature (Fig. 2.2 A&B). Oxygen saturation was recorded by oxygen micro-optodes.

For experiment 5, the larvae were transferred from the culture vessel into a plastic vessel, which was placed in a temperature-controlled seawater bath, containing seawater of the corresponding CO_2 condition. The plastic vessel was used for a careful introduction

of the larvae into the barrel of the Hamilton syringe. Afterwards the plunger of the high precision syringe was inserted and the volume of the chamber was reduced to 50 µl. The needle of the micro-sensor was inserted into the syringe through the cannula and the sensitive tip of the optode was placed in the middle of the chamber. The syringe was placed into the temperature-controlled seawater bath. Respiration measurements were stopped when the oxygen saturation of the chamber water reached about 80 %. Before and after two measurements, blanks were run to consider bacterial oxygen consumption.

For experiments 6, 7 and 8, a small batch of eggs was removed from the brooding female and placed on a fine grid in the respiration chamber. A magnetic stirrer was placed beneath the grid and the water in the respiration chamber was gently mixed to prevent oxygen stratification. The plunger of the chamber lid was inserted and the volume of the chamber was reduced to 250 µl. For measurements of larvae, individuals were carefully transferred from the culture vessel into the respiration chamber. Afterwards the plunger of the chamber lid was inserted and the volume of the chamber was reduced to 150 µl. The needle of the micro-sensor was inserted into the chamber through a hole in the lid and the sensitive tip of the optode was placed in the middle of the chamber. The swimming of the larvae caused mixing of the water in the chamber. Respiration measurements were stopped after thirty minutes or when the oxygen saturation of the chamber water had decreased to about 80 %. Before or after each measurement, blanks were run to correct for bacterial oxygen consumption.

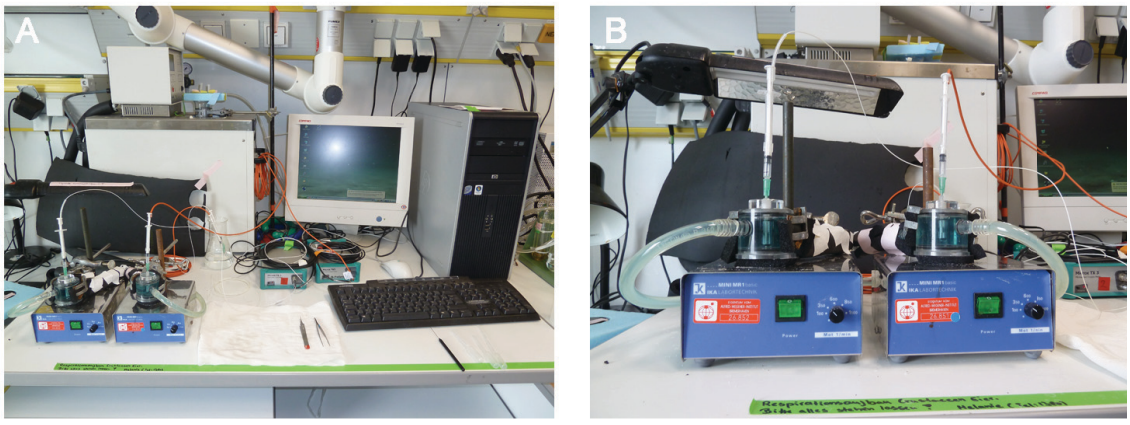


Fig. 2.2 A) Experimental setup for oxygen consumption measurements of larvae and eggs of *Hyas araneus*. B) Temperature-controlled double-walled respiration chambers.

2.3.4 Larval heart rate and maxilliped beat rate

Beside oxygen consumption rates, heart rate and maxilliped beat rates were measured in different larval stages of *Hyas araneus* at different temperatures to assess larval thermal tolerance windows (see publication III for details).

Heart rate and maxilliped activity were determined by using a digital camera (AxioCam MRm, Carl Zeiss, Mikroimaging GmbH, Göttingen, Germany) mounted onto an inverse microscope (Axio Observer A1, Carl Zeiss) (Fig. 2.3 A&B). Larvae were measured under the microscope in a custom-built temperature-controlled flow-through micro-chamber (AWI workshop) with a flow rate of 5 ml min^{-1} to avoid a decrease in oxygen concentration due to larval respiration in the closed chamber. Temperature controlled seawater was provided from a reservoir vessel placed in the thermostat's water bath and was pumped through the chamber. Before closing the chamber, individual larvae were positioned in the centre of the micro-chamber by gluing the carapace to a thin glass spine, which itself was fixed to a glass table. Afterwards, the chamber was closed and water flow through the chamber was started. Larvae were left for 1 h to recover from handling stress and videotaped for 1 min. The video sequence was analysed for heartbeat and maxilliped activity, respectively, by counting the beats min^{-1}. The beating heart can easily be seen through the transparent carapace. Heart rate and maxilliped beat rate were calculated for each larvae as means of beats per minute from three 10 s intervals.

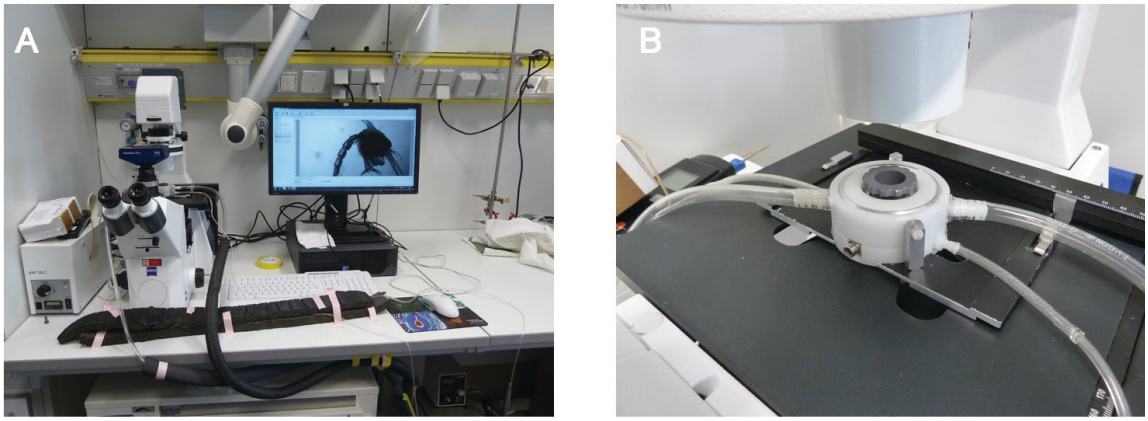


Fig. 2.3 A) Experimental set-up to measure heart rate and maxilliped beat rate of larval *Hyas araneus*. B) Temperature-controlled flow-through micro-chamber mounted onto an invers microscope.

2.4 Haemocyanin oxygen binding characteristics

A modified, temperature-controlled gas diffusion chamber (Sick and Gersonde 1969, Oellermann et al. in prep) connected to an Eppendorf photometer (model 1100M)

was used for determination of haemocyanin oxygen binding characteristics of adult *Hyas araneus*. Frozen haemolymph samples of *Hyas araneus* were thawed on ice, spun down at 10.000g for 10 min and 15 µl of the supernatant was applied to a sample glass plate. A fibre optic micro-pH optode was fixed onto the plastic slide that holds the sample glass plate in the light tunnel and the optode sensor tip was moved into the edge of the haemolymph droplet to reduce bleaching by the light beam in the middle of the glass plate. The glass holder was inserted and fixed in the diffusion chamber. A gas-mixing pump (Wösthoff, Germany) supplied an adjustable mixture of nitrogen, oxygen and carbon dioxide gas, via gas tight hoses. The haemolymph sample was exposed to pure oxygen to guarantee a complete oxygen saturation of haemocyanin and absorbance was measured at 348 nm. Subsequently, haemolymph samples were exposed to a defined mixture of oxygen (21, 12, 5, 2 and 1 kPa), nitrogen gas and an increased carbon dioxide concentration (10 kPa) to decrease the pH until a minimum in absorbance was reached. Afterwards, haemolymph samples were exposed to pure nitrogen to guarantee a complete desaturation of haemocyanin and absorbance was measured again. Absorbance measured during haemolymph exposure to pure oxygen and pure nitrogen was used for calibration.

2.5 Acid base parameter

2.5.1 Extracellular pH

Around 1 ml of haemolymph of adult *Hyas araneus* was obtained at the coxa of the third walking leg using a sterile syringe. Haemolymph was transferred to an Eppendorf tube (1.5 ml), placed in a thermostat water bath and pH was measured immediately at acclimation temperature with a pH meter (PHM 93, Radiometer Copenhagen, Electrode: InLab Micro, Mettler Toledo GmbH, Germany). The pH electrode was calibrated with NIST buffers.

2.5.2 Egg pH

Measurement of egg pH was carried out using the homogenization technique described by Pörtner (1990). Egg batches were removed from brooding female, directly frozen in liquid nitrogen and stored at -80°C until usage. For pH measurements, tissue samples were ground under liquid nitrogen by mortar and pestle and added to the buffer containing 2 mM nitrilotriacetic acid (NTA) and 320 mM potassium fluoride (KF). Homogenate pH was measured using a fibre optic micro-pH optode.

2.5.3 Calculation of P_{CO_2} and HCO_3^-

The total dissolved inorganic carbon content (CCO_2) of intra- and extracellular fluids were analysed according to the gas chromatographic method of Lefant and Aucutt (1966), which has been previously modified by Boutilier et al. (1985) and Pörtner et al. (1990). Homogenate (section 2.5.2) and extracellular fluid were injected into gas tight glass vials containing 3 ml of air equilibrated 0.1 M HCl and analysed by gas chromatography (Agilent 6890 N GC System, Agilent Technologies, USA). P_{CO_2} and bicarbonate [HCO_3^-] of extra- and intracellular fluids was calculated from CCO_2 and pH using the following forms of the Henderson-Hasselbalch equation:

$$P_{CO_2} = CCO_2 / (10^{pH-pKIII} * \alpha+\alpha)$$

$$[HCO_3^-] = CCO_2 - \alpha * P_{CO_2}$$

with

CCO_2 = total CO_2 concentration [mM]
αCO_2 = physical solubility of CO_2
P_{CO_2} = partial pressure of CO_2 [kPa]
pK= apparent dissociation constant of the CO_2/apparent [HCO_3^-] system

αCO_2 and pK were calculated according to Pörtner et al. (2010b).

Seawater physicochemistry was monitored by measuring temperature, salinity, pH (NBS scale, pH_{NBS}, corrected by Tris buffered seawater (Dixon lab, Scripps Institution of Oceanography, La Jolla, CA, USA)) and dissolved inorganic carbon (DIC). Seawater temperature and salinity were measured with a conductivity meter (WTW conductivity meter ProfiLine Cond 1970i). The pH measurements were conducted with a pH meter (WTW portable pH meter ProfiLine pH 3310) calibrated with NIST buffers. DIC was determined with Seal Analysis SFA QuAAtro; pump Technicon trAAcs 800 TM. Seawater P_{CO_2} was calculated from DIC, pH_{NBS}, temperature and salinity using the program CO_2SYS (Lewis and Wallace, 1998).

2.6 Female brooding behaviour

In order to examine whether eggs are oxygen limited due to less oxygen supply by the females at elevated seawater P_{CO_2}, females of *Hyas araneus* were videotaped and abdominal flapping was measured. Experiments were conducted at the aquaria facilities of

the Alfred Wegener Institute at constant room and seawater temperature. Ovigerous females of *Hyas araneus* with late stage III embryos were placed individually in closed transparent 2 l plastic tanks. A constant water flow was generated from the flow-through aquaria used for CO_2 incubation of the ovigerous females. The behaviour of each female was videotaped continuously over a period of 24 h at a constant dark: light cycle (12 h: 12 h) with an infrared light turned on during night. The frequency of occurrence of abdominal flapping events (n events per hour) and the number of abdominal flaps (n flaps per hour) were recorded and six to seven hours of video were analysed. Female behaviour was only analysed during night (Fig. 2.4).

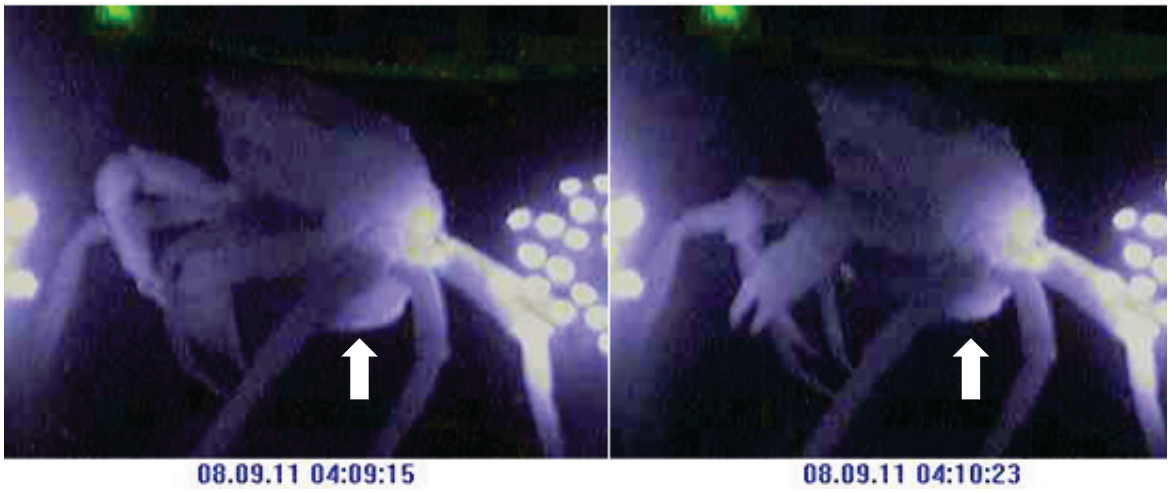


Fig. 2.4 Abdominal flapping performed by ovigerous females of *Hyas araneus* videotaped during the night.

2.7 Quantitative gene expression analysis

Quantitative gene expression analysis was used to measure the expression of various genes responsible for cellular stress/heat shock response, acid-base regulation and mitochondrial energy metabolism in different larval stages of *Hyas araneus* (publication III). Results will be used to determine if these processes are of central importance for a possible limitation in thermal tolerance during hypercapnic exposure. The same genes relevant for acid-base regulation were also measured in embryos and adults of *H. araneus* to compare life stage specific capacities of acid-base regulation on a molecular level.

For quantitative gene expression analysis, samples of larvae and embryos were transferred into 1.5 ml Eppendorf tubes containing RNA later (Ambion, Austin, TX) and stored at -80°C. Gill tissue of adult crabs was immediately frozen in liquid nitrogen and

stored at -80°C. The same protocol for mRNA extraction, cDNA synthesis and quantitative PCR was used for embryos, larvae and adult tissue (see material and method in publication III).

3 Publications

List of publications and declaration of my contribution towards them:

Publication I

Schiffer M, Harms L, Pörtner HO, Lucassen M, Mark FC and Storch D (2012). Tolerance of *Hyas araneus* zoea I larvae to elevated seawater P_{CO_2} despite elevated metabolic costs. Marine Biology DOI 10.1007/s00227-012-2036-0

I developed the concept and design of this study together with DS. I carried out the measurements and analysed the data. The manuscript was written by myself and was revised by all co-authors.

Publication II

Schiffer M, Harms L, Pörtner HO, Mark FC and Storch D. Pre-hatching seawater P_{CO_2} affects development and survival of the zoea stages of the Arctic spider crab *Hyas araneus*. Marine Ecology Progress Series (submitted)

The ideas for the experiments were developed by DS and myself. The experiments were conducted and analysed by myself. The manuscript was written by myself and revised by all co-authors.

Publication III

Schiffer M, Harms L, Lucassen M, Mark FC, Pörtner HO and Storch D. Temperature tolerance of different larval stages of the spider crab *Hyas araneus* exposed to elevated seawater P_{CO_2}

I developed the concept of this study together with DS and FCM. I developed the experimental design and conducted the experiments. I carried out the laboratory work together with LH. I analysed the data and wrote the manuscript, which was revised by all co-authors.

Additional publication

Harms L, Frickenhaus S, **Schiffer M**, Mark FC, Storch D, Pörtner HO, Held C and Lucassen M. Characterization and analysis of a transcriptome from the Arctic spider crab *Hyas araneus*. PLoS ONE (submitted)

I supported the experiments and helped revise the manuscript, which was written by the first author.

Publication I

Tolerance of *Hyas araneus* zoea I larvae to elevated seawater PCO_2 despite elevated metabolic costs

Schiffer M, Harms L, Pörtner HO, Lucassen M, Mark FC and Storch D

2012

Marine Biology

Mar Biol
DOI 10.1007/s00227-012-2036-0

ORIGINAL PAPER

Tolerance of *Hyas araneus* zoea I larvae to elevated seawater PCO_2 despite elevated metabolic costs

Melanie Schiffer · Lars Harms · Hans O. Pörtner · Magnus Lucassen · Felix C. Mark · Daniela Storch

Received: 21 December 2011 / Accepted: 2 August 2012

Abstract Early life stages of marine crustaceans respond sensitively to elevated seawater PCO_2. However, the underlying physiological mechanisms have not been studied well. We therefore investigated the effects of elevated seawater PCO_2 on oxygen consumption, dry weight, elemental composition, median developmental time (MDT) and mortality in zoea I larvae of the spider crab *Hyas araneus* (Svalbard 79°N/11°E; collection, May 2009; hatch, December 2009). At the time of moulting, oxygen consumption rate had reached a steady state level under control conditions. In contrast, elevated seawater PCO_2 caused the metabolic rate to rise continuously leading to a maximum 1.5-fold increase beyond control level a few days before moulting into the second stage (zoea II), followed by a pronounced decrease. Dry weight of larvae reared under high CO_2 conditions was lower than in control larvae at the beginning of the moult cycle, yet this difference had disappeared at the time of moulting. MDT of zoea I varied between 45 ± 1 days under control conditions and 42 ± 2 days under the highest seawater CO_2 concentration. The present study indicates that larval development under elevated seawater PCO_2 levels results in higher metabolic costs during premoulting events in zoea I. However, *H. araneus* zoea I larvae seem to be able to compensate for higher metabolic costs as larval MDT and survival was not affected by elevated PCO_2 levels.

Communicated by S. Dupont.

M. Schiffer (✉) · L. Harms · H. O. Pörtner · M. Lucassen · F. C. Mark · D. Storch
Department Integrative Ecophysiology, Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany
e-mail: Melanie.Schiffer@awi.de

Introduction

Since colder seawater displays higher solubility for CO_2, it has been predicted that the largest pH changes due to absorption of atmospheric CO_2 over the twenty-first century will occur in the Arctic Ocean. Furthermore, the Arctic Ocean is predicted to become increasingly undersaturated with respect to aragonite and calcite in the coming decades (Steinacher et al. 2009), which might especially affect calcifying Arctic species (Lischka et al. 2010).

Various effects of elevated PCO_2 on the physiology of marine organisms have already been described. Affected processes include calcification, but also acid–base regulation and metabolism, growth and reproduction (Melzner et al. 2009). It is known that elevated seawater PCO_2 leads to decreasing extracellular pH in crustaceans (Cameron 1978; Wheatly and Henry 1992; Pane and Barry 2007). There is also evidence that an uncompensated drop in extracellular pH has a depressing effect on aerobic energy metabolism of some tissues like muscle (Reipschläger and Pörtner 1996) and isolated liver cells (Langenbuch and Pörtner 2003), through effects on the mode and rate of proton equivalent ion exchange (Pörtner et al. 2000) and a decrease in protein synthesis (Langenbuch et al. 2006). This may result in decreasing whole animal oxygen consumption (Pörtner et al. 1998; Michaelidis et al. 2005) or, if compensated for by the rise in energy demanding processes (e.g. calcification), an increase in whole organism oxygen demand (Thomsen and Melzner 2010, Lannig et al. 2010; Stumpp et al. 2011) and an associated shift in energy budgets. So far, it is not known to what extent these patterns also hold for early life stages of crustaceans, which have to allocate considerable amounts of energy to growth and morphological changes and may be the more sensitive life stages under ocean acidification (Walther et al. 2010).

Published online: 26 August 2012

Therefore, assessing energy turnover during larval development under elevated seawater PCO_2 may help to predict possible consequences of ocean acidification for this marine group.

Adult crustaceans seem to be relatively tolerant of ocean acidification, presumably due to an expansive ability to compensate for acid–base disturbances. In two efficiently ion-regulating prawn species, extracellular acidosis was fully compensated after 30 days of exposure to a PCO_2 of 3,000 µatm (Dissanayake et al. 2010). Also poor ion-regulators like the decapod species *Necora puber* and *Cancer magister* were able to recover their haemolymph pH over 24 h at a seawater PCO_2 of 8,000 µatm (Pane and Barry 2007, Spicer et al. 2007). However, crustacean species adapted to a stable environment like the deep sea seem to be unable to compensate haemolymph acid–base disturbances during hypercapnia and might therefore be especially vulnerable to ocean acidification (Pane and Barry 2007).

While early life stages of crustaceans may be more sensitive to high seawater PCO_2 levels than adults, the physiological background of their sensitivity has not been investigated. An increase in larval mortality and a decrease in hatching success under elevated seawater PCO_2 have been observed in two species of marine copepods (Kurihara et al. 2004). Other crustacean larvae showed altered growth and calcification rates at high seawater CO_2 concentrations at 1,200–3,000 µatm (Arnold et al. 2009, Walther et al. 2010, 2011). Growth rates were also reduced in early life stages of the barnacle *Semibalanus balanoides* reared at 1,000 µatm (Findlay et al. 2010). In larvae from two populations of the spider crab *Hyas araneus*, extended development times in zoea I and zoea II were recorded at low temperatures of 3 °C and the highest CO_2 treatment of 3,000 µatm CO_2, while elevated PCO_2 values reduced larval growth in megalopa but not in zoea I and zoea II (Walther et al. 2010). By sampling once per larval stage, the authors concluded that the megalopa stage might be a physiologically sensitive bottleneck. However, total larval development was delayed and the megalopa stage reached with some time delay under elevated CO_2. Larval weight, elemental composition and energy demand vary with age (Anger et al. 1989) within and between each life stage (Arnold et al. 2009; Walther et al. 2010, 2011). Developmental timing events may also vary between CO_2 treatments, and therefore several time points should be used for such comparisons to avoid misleading conclusions (Pörtner et al. 2010).

Development in crustaceans is characterised by regular moulting events including metamorphosis. From moult to moult, the larvae undergo growth and/or morphological changes (Anger 1983), which are accompanied by changing rates of oxygen consumption depending on larval stage, day of development and, accordingly, day within the moulting cycle (Anger et al. 1989; Anger 2001). Metabolic requirements of crustacean larval development with respect to abiotic factors like temperature and salinity have been studied in detail (Jacobi and Anger 1985; Anger et al. 1998). However, ongoing ocean acidification may entail new challenges for successful development. During their complex life cycle, crustaceans face variable environmental conditions. The first larval stages (zoea larvae) are pelagic and most of them are actively swimming in the upper water column. The megalopa larvae mark the transition from the pelagic to the benthic mode of life. Since Arctic surface waters are predicted to undergo larger pH changes than other oceanic regions (Steinacher et al. 2009), the early larval stages of Arctic crustaceans could be among the first to show significant effects of ocean acidification.

We therefore investigated the effect of elevated CO_2 levels on energy turnover during development of zoea I of the spider crab *H. araneus* from an Arctic population of Svalbard (Norway). *H. araneus* is a continental shelf species, which inhabits rocky, sandy and muddy bottoms and has a wide distribution range from the temperate southern North Sea to subarctic waters (Christiansen 1969). Broad knowledge exists on its larval development depending on several abiotic conditions (Anger 2001; Walther et al. 2010, 2011). This makes it an adequate model organism to study the effects of elevated seawater PCO_2 on the development of crustacean larvae. Ovigerous females carry the egg masses for approximately 2 years (Petersen 1995). After the embryonic phase, zoea I hatch from the eggs and are released into the water column. Larvae undergo two zoeal stages, and one megalopa stage before settling as juveniles in the adult habitat. We investigated potential changes in sensitivity and the specific patterns of physiological response during the time course of the first larval stage, the zoea I. We followed the course of oxygen consumption, weight and elemental composition in developing zoea I larvae of Arctic *H. araneus* and determined mortality and median developmental time (MDT) under different CO_2 levels. With our data, we have been able to distinguish age-related effects from the effects of elevated CO_2 levels.

Materials and methods

Larval collection and maintenance

Ovigerous females of Arctic *H. araneus* were collected in Kongsfjorden (Ny Alesund, Svalbard 78°55 N, 11°56 E) by scientific divers during May 2009 and transferred to the Alfred Wegener Institute in Bremerhaven. They were

maintained in flow-through aquaria at 4 °C, which corresponds to the seawater temperature in the Kongsfjorden during summer, 32 psu and a constant dark/light cycle (12 h:12 h). Four females were placed individually in 2 l flow-through aquaria shortly before larval hatching. Larvae hatched during December 2009. Equal numbers of newly hatched larvae of the four females were pooled and subsequently transferred into 0.5 l culture vessels at a density of 30 individuals per vessel. Experiments were conducted with zoea I larvae that had hatched within 24 h. They were reared in 27 culture vessels, respectively, filled with seawater of different CO_2 concentrations (27 culture vessels for each CO_2 treatment: 490 µatm, 1,100 µatm, 2,400 µatm CO_2) at a constant temperature of 6.2 ± 0.1 °C. Out of these 27 vessels, three vessels were used for mortality observation and developmental time, and individuals from the remaining 24 vessels were used for oxygen consumption, dry weight and C:N ratio measurements. Seawater was provided from reservoir tanks (60 l) kept at the same temperature as culture vessels and bubbled with air/CO_2 mixture using a mass flow controller (HTK 6 channel, HTK Hamburg GmbH, Germany). Water in culture vessels was changed daily, and dead larvae and moults were removed. Larvae were fed daily ad libitum with freshly hatched *Artemia* sp. (Sanders Brine Shrimp Company, Ogden, Utah, USA). Days of development and age of larvae are given as days post hatching (dph). Water physicochemistry was monitored weekly by determinations of pH and the collections of water samples for the determination of dissolved inorganic carbon (DIC) and total alkalinity. Water PCO_2 was calculated from DIC and pH_{NBS} using the program CO_2 SYS (Lewis and Wallace 1998, Table 1).

Mortality and median developmental time (MDT)

Three culture vessels per CO_2 concentration (of about 30 larvae each) were used for investigating the effect of elevated CO_2 on the mortality and MDT of the first larval stage. Mortality and moulting (number of zoea II) were recorded on a daily basis until all larvae were either dead or moulted into the zoea II. Dead larvae and zoea II were removed. Larval daily mortality and total mortality were calculated for each vessel and expressed as percentage. For larval daily mortality, the relative (percentual) proportion refers to number of zoea I remaining after each day. Culture vessels were used as replicates, and total mortality was determined as means (±SE) of the three replicates.

Median developmental time was calculated according to Landry (1983). Age was measured from the day of development the larvae started hatching, and MDT is defined as the time when 50 % of the zoea I larvae have moulted to zoea II. It was estimated from least-square regressions of the cumulative proportion of all larvae that had not yet passed the zoea I stage plotted against time. MDT was obtained from the exponential part of the moulting curve, and data below the 40 % moulting level were left out. MDT for larvae of corresponding seawater CO_2 concentration was determined as means of the three replicates. Moulting range is given as first day when zoea I started moulting until the day when the last zoea I moulted to the second stage.

Oxygen consumption

Oxygen consumption rates of individual zoea I were measured according to Storch et al. (2009). Hamilton high-precision microlitre syringes (500 µl, Hamilton-Bonaduz, Switzerland) were used as closed respiratory chambers. Oxygen saturation was recorded by oxygen micro-optodes (needle-type NTH-PSt1-L5-TF-NS55x0,80-PC3,1-YOP, fibre-optic microsensor, flat broken tip, diameter: 140 µm, PreSens GmbH, Regensburg, Germany), connected to a 4-Channel Microsensor Oxygen Meter (PreSens GmbH, Regensburg, Germany). The larvae were transferred from the culture vessel into a plastic vessel, which was placed in a temperature-controlled seawater bath, containing seawater of the corresponding CO_2 condition. The plastic vessel was used for a careful introduction of the larvae into the barrel of the Hamilton syringe. This handling took place under water to avoid air bubbles. Afterwards the plunger of the high-precision syringe was inserted, and the volume of the chamber was reduced to 50 µl. The needle of the microsensor was inserted into the syringe through the cannula, and the sensitive tip of the optode was placed in the middle of the chamber. The syringe was placed into the temperature-controlled seawater bath (6 °C). Respiration

Table 1 Seawater parameters measured during incubation

Incubation	Temperature (C°)	pH_{NBS}	DIC (µmol/kg)	Alkalinity (µmol/kg)	P_{CO2} (µatm)
Control	6.13 ± 0.17	8.10 ± 0.03	2320 ± 63	2417 ± 6	489 ± 21
CO_2 1100	5.96 ± 0.24	7.80 ± 0.04	2435 ± 33	2482 ± 6	1026 ± 107
CO_2 2400	6.3 ± 0.13	7.44 ± 0.02	2524 ± 77	2483 ± 5	2343 ± 130

Values are given in mean ± SD. $N = 9$

NBS National Bureau of Standards, *DIC* dissolved inorganic carbon, PCO_2 partial pressure of CO_2

measurements were stopped when the oxygen saturation of the chamber water reached about 80 %. Before and after two measurements, blanks were run to consider bacterial oxygen consumption. Oxygen consumption was expressed as $\mu gO_2/Ind^{-1}\ h^{-1}$ as there was no correlation with larval dry weight. At all sampling days, eight larvae from each CO_2 treatment were used to measure oxygen consumption. The same eight individuals were used for dry weight and C:N ratio measurements. For each day of measurement, eight larvae were taken from a particular vessel. Vessels were sampled between one and four times throughout the experiment (i.e. on one to four different days).

Dry weight and C:N ratio

After respiration measurements, all larvae were removed from the chamber and briefly rinsed with deionised water. Excessive water was removed using a paper towel. Subsequently, larvae were stored at −20 °C in preweighed tin cartridges for analyses. Individual larvae within the tin cartridges were freeze-dried over night, weighed on a high-precision balance (Mettler Toledo AG, Greifensee, CH-8606, CH), and C:N ratios were measured in a CN analyzer (Euro EA-CN analyzer), using acetanilide as standard. C and N are given as µg per individual and as percent of dry weight (%DW). Dry weight is given as µg per individual. Unfortunately, no data on dry weight and C and N content could be obtained for the first 8 days of development in the present study due to loss of samples.

Statistical analyses

Results were analysed using GraphPad Prism 4 (GraphPad Software Inc.). All data were checked for outliers by use of Nalimov's test (Noack 1980). A two-way ANOVA was used to investigate the effects of CO_2 concentration and larval age on larval oxygen consumption, dry weight, C and N content and C:N ratio. Bonferroni tests were used for a posterior analyses. When the interaction between factors was significant, an a posterior Bonferroni test was run separately for each CO_2 concentration to detect differences among days of development. A one-way ANOVA was conducted to analyse the effect of CO_2 on MDT and daily mortality of larvae, followed by an a posterior Bonferroni test.

Results

Mortality and median developmental time (MDT)

There was no effect of CO_2 on daily mortality (ANOVA: $F_{2,35} = 1{,}131$; $p = 0.3349$) and on total mortality (ANOVA: $F_{2,8} = 0{,}2090$; $p = 0.8170$). Mean daily mortality was 0.3 ± 0.7 % in larvae exposed to 490 µatm CO_2, 0.2 ± 0.6 % in larvae exposed to 1,100 µatm and 0.2 ± 0.6 % in larvae exposed to 2,400 µatm CO_2, while total mortality was 18.7 ± 2.3 % in larvae exposed to 490 µatm CO_2, 17.5 ± 9.5 % in larvae exposed to 1,100 µatm CO_2 and 13.4 ± 4.0 % in larvae from the 2,400 µatm CO_2 treatment.

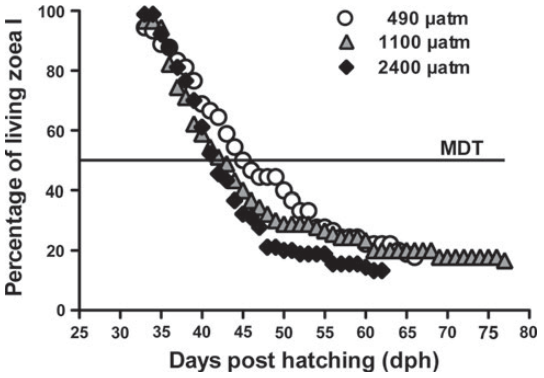


Fig. 1 Percentage of living zoea I larvae of *H. araneus* reared under three different seawater PCO_2 (490 µatm *white circles*, 1,100 µatm *grey triangle*, 2,400 µatm *black rectangle*) during time of development. *Line* indicates time when 50 % of the zoea I larvae have moulted to zoea II (median developmental time, MDT)

Seawater CO_2 concentration had no effect on the MDT of the first larval stage (ANOVA: $F_{2,8} = 1{,}217$; $p = 0.3600$). MDT of zoea I at 490 µatm CO_2 was 45 ± 1 days and 43 ± 4 at 1,100 µatm CO_2, whereas larvae exposed to 2,400 µatm CO_2 moulted on day 42 ± 2 (Fig. 1). First moulting into zoea II occurred at day 33 in all three CO_2 treatments but moulting ranged between day 33 and day 66 in larvae reared at 490 µatm CO_2, between day 33 and day 77 in larvae reared at 1,100 µatm CO_2 and between day 33 and day 62 in larvae reared at 2,400 µatm CO_2 (Fig. 1).

Oxygen consumption

Oxygen consumption varied greatly with developmental time but in different ways for the three CO_2 treatments (Fig. 2a–c). Oxygen consumption rates in larvae reared at 490 µatm CO_2 (controls) decreased during the first few days after hatching, increased during further development and reached a steady state level before moulting. In contrast, oxygen consumption of larvae reared under higher CO_2 conditions followed a similar pattern during the first days but showed a significant increase in oxygen consumption followed by a pronounced decrease a few days before moulting. Consequently, the 2-way ANOVA showed a significant interaction between day of development and

CO_2 concentration (ANOVA II: $F_{28,289} = 2{,}107; p = 0.0013$) (Table 2).

The Bonferroni test showed a significant increase in oxygen consumption at 490 µatm CO_2 between 2- and 22-day-old larvae ($p < 0.05$) followed by a constant rate in 22–42-day-old larvae ($p > 0.05$, Fig. 2a). Oxygen consumption rates from larvae incubated at 1,100 µatm CO_2 also increased significantly between day 2 and day 22 ($p < 0.05$, Fig. 2b), remained constant between day 22 and day 27 ($p > 0.05$) and increased again significantly between day 27 and day 36 ($p < 0.001$), where oxygen consumption peaked at 0.234 ± 0.01 $\mu gO_2/Ind^{-1}\ h^{-1}$ followed by a significant decrease on day 42 ($p < 0.001$, Fig. 2b). A similar trend could be found in larvae incubated at 2,400 µatm CO_2 (Fig. 2c). Oxygen consumption rates increased significantly between day 4 and day 24 ($p < 0.05$), levelled off between day 24 and 34 ($p > 0.05$) and increased again on day 36 ($p < 0.001$). A significant decline in oxygen consumption rates on day 42 ($p < 0.001$) was observed. At 2,400 µatm CO_2, the highest oxygen consumption rate was found on day 36 (0.231 ± 0.03 $\mu gO_2/Ind^{-1}\ h^{-1}$, Fig. 2c). An unpaired t test showed a significant difference between oxygen consumption rates of the three treatments on day 36, with higher oxygen consumption in larvae from each CO_2 treatment compared with those of control larvae (unpaired t test: 1,100 µatm CO_2: $p < 0.05$; 2,400 µatm CO_2: $p < 0.05$, Fig. 2a–c).

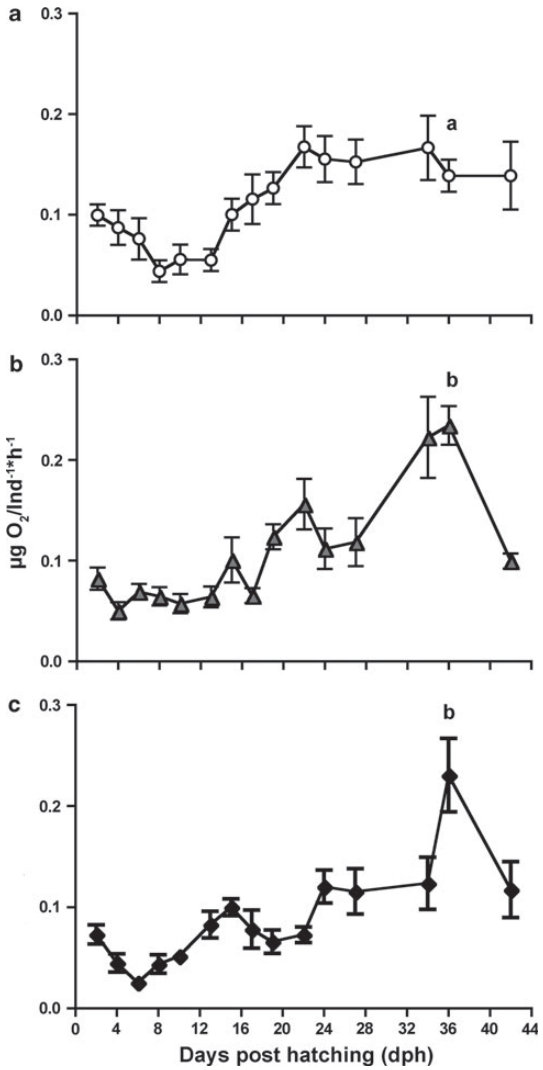


Fig. 2 Individual oxygen consumption during development of zoea I larvae of *H. araneus* reared under three different levels of seawater PCO_2 (**a** 490 µatm, **b** 1,100 µatm, **c** 2,400 µatm). Different l*owercase letters* indicate significant differences between treatments on the same developmental day. Mean ± SE

Dry weight, C:N ratio, C + N (%) of dry weight

Changes in larval dry weight over time followed bell-shaped curves in all three treatments (Fig. 3). There was a significant interaction detected by 2-way ANOVA between larval age and CO_2 concentration (ANOVA II: $F_{14,155} = 2{,}064$; $p = 0.0166$) (Table 2). The Bonferroni test showed two peaks in dry weight in larvae exposed to 490 µatm CO_2. Dry weight increased between day 8 and 22 ($p < 0.001$), decreased on day 24 ($p < 0.001$) and remained constant between day 24 and 34 ($p > 0.05$). Subsequently, dry weight rose on day 36 ($p < 0.05$) followed by a significant drop in weight by day 42 ($p < 0.001$) (Fig. 3). Dry weight of larvae from incubations under 1,100 and 2,400 µatm CO_2 increased significantly between day 8 and 22 ($p < 0.001$) and remained constant thereafter. Larvae under 2,400 µatm CO_2 displayed constant dry weight between day 22 and 36 ($p > 0.05$), followed by a decrease on day 42 ($p < 0.01$). In larvae under 1,100 µatm CO_2, the decline could already be seen on day 27 ($p < 0.001$). An unpaired t test showed that on day 8, dry weight of larvae under 2,400 µatm CO_2 remained significantly below that of larvae under 490 µatm CO_2 and 1,100 µatm CO_2 (unpaired t test: 490 µatm CO_2: $p < 0.05$; 1,100 µatm CO_2: $p < 0.001$, Fig. 3).

While CO_2 had no direct effect, larval age significantly affected C and N contents of zoea I larvae (ANOVA II: C: $F_{7,165} = 19{,}43$; $p < 0.0001$; N: $F_{7,166} = 22{,}56$; $p < 0.0001$; Table 2, Fig. 4a, b). In larvae exposed to 490 µatm CO_2, C content increased between day 19 and 22 ($p < 0.01$), remained constant until day 34 and increased again on day 36 ($p < 0.01$) followed by a significant drop on day 42 ($p < 0.001$). In larvae from both high CO_2 treatments, C content increased significantly between day 8

Table 2 Results of 2-way ANOVAs conducted to investigate effects of CO_2 and larval age on oxygen consumption, dry weight, carbon content (C), nitrogen content (N), C:N ratio and weight-specific sum of C and N of *H. araneus* zoea I larvae

Response variable	CO_2 effect			Day of development			Interaction		
	F	*df*	*p*	*F*	*df*	*p*	*F*	*df*	*p*
Individual oxygen consumption	5,519	2	**0.0044**	15.73	14	**<0.0001**	2,107	28	**0.0013**
Dry weight	7,691	2	**0.0007**	40.21	7	**<0.0001**	2,064	14	**0.0166**
C	1,227	2	0.2959	19.43	7	**<0.0001**	1,742	14	0.0518
N	0,413	2	0.6618	22.56	7	**<0.0001**	1,516	14	0.1100
C:N	2,225	2	0.1113	13.30	7	**<0.0001**	6,816	14	**<0.0001**
Sum of weight-specific C and N	6,065	2	**0.0029**	11.10	7	**<0.0001**	3,671	14	**<0.0001**

Significant values with $p < 0.05$ are given in bold

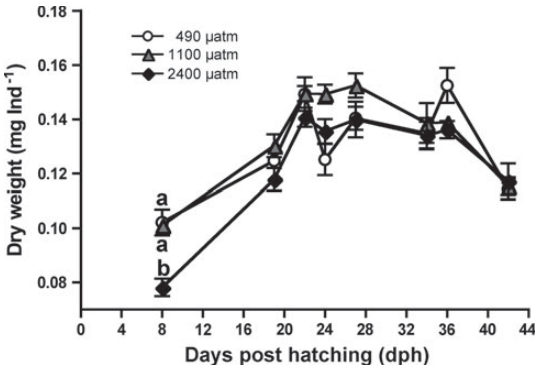


Fig. 3 Dry weight of zoea I larvae of *H. araneus* reared under three different seawater PCO_2 (490 µatm *white circles*, 1,100 µatm *grey triangle*, 2,400 µatm *black rectangle*) during time of development. *Different letters* indicate significant differences between treatments on the same developmental day. Mean ± SE

and 19 ($p < 0.01$), levelled off between day 19 and 36, and dropped on day 42 (1,100 µatm CO_2: $p < 0.01$; 2,400 µatm CO_2: $p < 0.05$, Fig. 4a). In all treatments, N content increased significantly between day 8 and 22 ($p < 0.001$, Fig. 4b). Afterwards it remained constant and finally decreased between day 36 and 42 in larvae exposed to 490 µatm CO_2 ($p < 0.01$) and between day 34 and 42 in larvae exposed to 1,100 µatm CO_2 ($p < 0.001$), respectively. In larvae exposed to 2,400 µatm CO_2, a stable N content could be found between day 22 and 42.

There was a significant interaction in the 2-way ANOVA between larval age and CO_2 concentration on larval C:N ratio (ANOVA II: $F_{14,166} = 6{,}816$; $p < 0.0001$, Ta. 2, Fig. 4c). Under 490 µatm CO_2, the C:N ratio decreased between day 8 and 19 ($p < 0.001$) and again between day 22 and 24 ($p < 0.001$). It remained constant between day 24 and 34 and finally increased between day 34 and 36 ($p < 0.001$). In larvae from 1,100 µatm CO_2, the C:N ratio stayed constant between day 8 and 34, followed by an increase between day 34 and 36 ($p < 0.001$) and a drop on day 42 ($p < 0.01$). Under 2,400 µatm CO_2, an increase in the C:N ratio could be found between day 8 and 19 ($p < 0.001$), followed by a decrease on day 22 ($p < 0.001$) and a constant C:N ratio between day 22 and 42.

On the sum of weight-specific C and N content (Fig. 5a–c), there was a significant interaction in the 2-way ANOVA between larval age and CO_2 concentration (ANOVA II: $F_{14,154} = 3{,}671$; $p < 0.0001$, Table 2, Fig. 5a–c).

An unpaired *t* test showed a significant difference between the sum of C and N content (%DW) of the three treatments on day 8, with higher sum of C and N content (%DW) in larvae from 2,400 µatm CO_2 compared with those of 490 µatm CO_2 larvae ($p < 0.01$) and 1,100 µatm CO_2 larvae ($p < 0.001$) (Fig. 5a–c). On day 22, an unpaired *t* test revealed a significant reduced sum of C and N content (%DW) in larvae reared at 2,400 µatm CO_2 compared with larvae at 490 µatm CO_2 ($p < 0.05$) as well as a significantly higher sum of C and N content (%DW) on day 34 in larvae from each high CO_2 treatment compared with those of at the larvae at 490 µatm CO_2 (1,100 µatm CO_2: $p < 0.05$; 2,400 µatm CO_2: $p < 0.01$) (Fig. 5a–c).

Discussion

Our data demonstrate the importance to disentangle the effects of larval age from those of abiotic factors on physiological parameters during the analysis of time series of a single larval stage. This has been revealed by former studies on sea urchin larvae with continuous development (Stumpp et al. 2011; Martin et al. 2011), but to our knowledge, the present study is the first one on a species with discrete development. Larval age had a strong effect on all measured parameters in comparison with CO_2. Therefore, the transient increase in oxygen consumption caused by CO_2 might not have been detected by sampling only once during each developmental stage. Since the development of crustacean larvae involves drastic

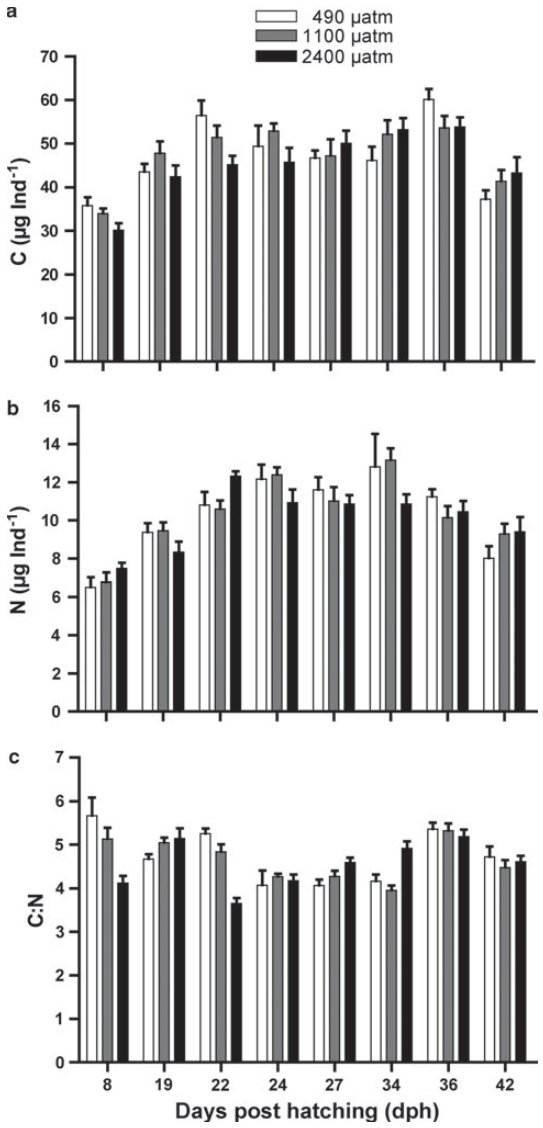


Fig. 4 Carbon content (**a**), nitrogen content (**b**) and C:N ratio (**c**) of *H. araneus* zoea I larvae reared under 490 µatm (*white*), 1,100 µatm (*grey*) and 2,400 µatm (*black*) during time of development. Mean ± SE

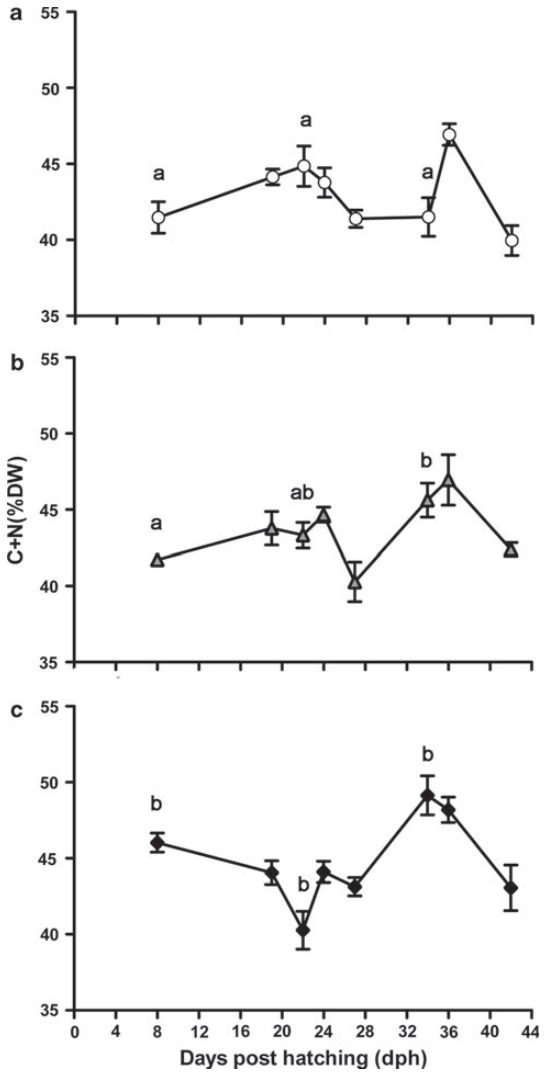


Fig. 5 Sum of carbon and nitrogen (% of dry weight) in *H. araneus* zoea I larvae reared under three different levels of seawater PCO_2 (**a** 490 µatm, **b** 1,100 µatm, **c** 2,400 µatm). *Different letters* indicate significant differences between treatments on the same developmental day. Mean ± SE

morphological changes, which can be classified in decapods according to Drach (1939), the high respiration rates at the end of the moult cycle suggest that the energy demand for morphological changes is enhanced by hypercapnic conditions. In *H. araneus* zoea I from Helgoland, the late premoult phase, which is characterised by the appearance of new cuticle, accounted for 26 % of total developmental time (Anger 1983). Assuming similar relationships in Svalbard zoea I, the premoult phase would set in approximately on day 31, followed by a peak in oxygen consumption under elevated CO_2. Thus, it is very likely that increased oxygen consumption in larvae reared under high seawater CO_2 concentrations indicates high metabolic costs associated with the appearance of the new cuticle.

Shortly before moulting, the larvae possess two cuticles, and the epidermis reaches its greatest retraction from the

old cuticle (Anger 1983). As respiratory gas exchange is mainly accomplished via the body surface in this larval stage, diffusion distances for respiratory gas (CO_2 and O_2) might be enlarged. A limited gas exchange would resemble hypoxic conditions and would cause the haemolymph O_2 level to decrease and CO_2 level to increase as it has been found in hypoxia studies (Varley and Greenaway 1992; Luquet and Ansaldo 1997). It is known that elevated seawater PCO_2 also leads to an increase in haemolymph PCO_2 in crustaceans (Pane and Barry 2007). Thus, it is likely that the zoea I larvae reared at 1,100 µatm and 2,400 µatm CO_2 experienced haemolymph acid–base disturbances during the premoult phase caused by a high extracellular PCO_2. Spicer and Eriksson (2003) found the thin cuticle of the telson to be the main site for respiratory gas exchange in early life stages of the Norway lobster *Nephrops norvegicus*. They found a correlation between pleopod activity, and seawater oxygen saturation with increasing pleopod activity at decreasing oxygen saturation. Storch et al. 2009 found higher oxygen consumption rates at increased maxilliped activity in zoea I of the Chilean kelp crab *Taliepus dentatus* under unfavourable low temperature. Enhanced maxilliped activity probably favours the gas exchange during unfavourable conditions like a limitation in oxygen supply. Thus, it is conceivable that increased oxygen consumption during the premoult phase in *H. araneus* zoea I larvae reared under high seawater CO_2 concentrations is due to enhanced larval activity to improve the haemolymph CO_2 release.

Growth and elemental composition are classic parameters to investigate effects of abiotic factors on the development of crustacean larvae (Anger et al. 1998). So far, a PCO_2-induced decrease in dry weight has only been found in late developmental stages (Arnold et al. 2009; Walther et al. 2010). Although overall larval growth and elemental composition were not affected by high seawater PCO_2 in the present study, a significant change in larval dry weight could be found in the zoea I on day 8 (Fig. 3) under the highest CO_2 concentration (2,400 µatm) when larval dry weight was considerably reduced. Interestingly, the difference was visible in dry weight only, whereas the elemental composition (C and N content) remained the same. Anger et al. (1989) found a strong increase in dry weight at the beginning of each moult cycle in *H. araneus,* while organic components (i.e. C and N) did not increase as rapidly. This leads to a decrease in the percentage of organic components in larval dry weight. The decrease in C and N at increasing dry weights was explained by an initial phase of rapid uptake of inorganic substances at the beginning of the moult cycles of all larval stages (zoea I, zoea II and megalopa; cf. Anger et al. 1989). This assumption is supported by an increased mineral (ash) content in percent of dry weight at the beginning of each moult cycle (Anger 2003). In the present study, we found a higher percentage of organic components in larval dry weight on day 8 only at the highest seawater CO_2 concentration of 2,400 µatm followed by an initial decrease in organic components in larval dry weight. Accordingly, it is likely that the low dry weight of zoea I on day 8 of development was due to a less pronounced uptake of inorganic substances in larvae reared under the highest CO_2 concentration as dry weight equalled with progressing development in all treatments, and no differences could be found on day 22. There might be an extended period of mineral uptake in larvae from the highest CO_2 concentration. This suggests a shift in the energetic balance. It is thus possible that zoea I have slowed down energy demanding processes like mineral uptake in favour of other processes like acid–base regulation.

In the Svalbard population of *H. araneus* from Kongsfjorden, zoea I larvae hatch between late February and early April (Walther et al. 2010). This corresponds to the Arctic spring bloom in the Kongsfjorden (Hop et al. 2002), which presents a sufficient food supply for small zooplankton. In crustaceans, different larval feeding strategies can be found. While larvae development of some crustacean species at high latitudes comprises a lecithotrophic phase (Anger et al. 2003), Arctic *H. araneus* larvae depend on food out of the water column for a successful development. Former studies indicated the crucial importance of a sufficient food supply for development and survival of crustacean larvae (Anger and Dawirs 1981; Dawirs 1983). Larvae in the present study were fed ad libitum to guarantee a suitable food supply, which should be consistent with natural food ability as the estimated biomass (dry mass) in Kongsfjorden is relatively high with 8.8 ± 5.1 g m^{-2} (Hop et al. 2002). However, larvae might be differently affected by elevated CO_2 when food supply is limited.

Measurements of larval dry weight should be a suitable indicator for growth and feeding in crustacean larvae to ensure adequate food supply. However, to date the only studies focusing on growth in *H. araneus* have been carried out with the Helgoland population at higher temperatures compared with the present study (Anger and Dawirs 1982; Anger and Jacobi 1985). The overall growth of Svalbard larvae during the zoea I stage followed a bell-shaped curve independent of seawater CO_2 concentration, while *H. araneus* zoea I from waters around Helgoland displayed almost exponential growth curves, and bell-shaped growth curves were only common in megalopa (Anger and Dawirs 1982; Anger and Jacobi 1985). A simple explanation could be the considerably longer developmental time of Svalbard zoea I compared with Helgoland zoea I. While it took 44 days under control conditions to moult into zoea II in the Svalbard population (6 °C), Helgoland zoea I need only

10 days at a rearing temperature of 12 °C. It is known that *H. araneus* zoea I feed continuously until the middle of the moult cycle (Anger et al. 1989), which can explain the significant increase in dry weight between day 8 and day 22. Afterwards larval feeding decreases. Assuming similar patterns in larvae from Svalbard, a longer period between the reduction in feeding and the moulting event might explain their bell-shaped growth curve. The almost exponential growth curve in Helgoland larvae is supported by their rapid development.

The temporary increase in oxygen consumption under elevated CO_2 was not accompanied by elevated mortalities or increased developmental time of zoea I larvae under both CO_2 conditions of 1,100 and 2,400 µatm. Although not significant, mortality and MDT decreased with increasing CO_2 concentration. However, the low number of statistical replicates might explain the lack of significance. This finding is in line with those of other ocean acidification studies on larval crustaceans. Mortality during zoea development of the shrimp *Pandalus borealis* also remained unaffected by elevated seawater PCO_2 of 1,300 µatm (Bechmann et al. 2011). Mortality of shrimp zoea during an experimental period of 35 days was in fact less under high seawater PCO_2 on the last day of experiment. In the copepod *Acartia tsuensis,* high seawater CO_2 concentration of about 2,300 µatm had no effect on survival or developmental speed through all life stages (Kurihara and Ishimatsu 2008). Also, survival of larval Decapoda seems to be largely unaffected by elevated seawater PCO_2. Survival and developmental time of all zoeal stages of the European lobster *Homarus gammarus* were not affected by seawater CO_2 concentration of 1,200 µatm (Arnold et al. 2009). However, the previous study on the larvae of the spider crab *H. araneus* demonstrated negative impacts on larval development (Walther et al. 2010). At a higher CO_2 concentration of 3,000 µatm and lower temperature of 3 °C than in our study they found an increased developmental time in both zoeal stages, but also no effect on mortality. The influence of elevated seawater PCO_2 on larval developmental time vanished at higher temperatures (Walther et al. 2010), which is in accordance with our findings.

In previous studies, effects of elevated PCO_2 on dry weight and mineral content in larvae of *H. gammarus* and *H. araneus* were only found in the latest stages (Arnold et al. 2009; Walther et al. 2010). Therefore, the megalopa stage of *H. araneus* was postulated to represent the larval stage most sensitive to high seawater PCO_2 (Walther et al. 2010). Our monitoring of the time course of zoea I development indicates that CO_2 effects may set in earlier, although at first sight, CO_2 acidified seawater had no negative effect on the successful development of *H. araneus* zoea I, as moulting into zoea II was similarly successful under all conditions. However, the course of oxygen consumption in developing zoea I indicates that development under elevated seawater PCO_2 level seemed to be more costly during premoulting. The effects of elevated seawater PCO_2 on dry weight and metabolism seen in the present study did not influence zoea I survival. It seems that the zoea I stage of *H. araneus* is able to compensate for the elevated costs associated with the development in a high PCO_2 environment. However, as it has been found by Dupont et al. (2012) this compensation might be an energy consuming process and might therefore affect performance of later stages by carry-over effects due to depletion of energy reserves.

Acknowledgments Financial support was provided by Federal Ministry of Education and Research (BMBF), within phase I of the BIOACID research programme (FKZ 03F0608B, subproject 2.2.1). We thank the scientific divers of the Alfred Wegener Institute for animal collection and C. Lorenzen for technical assistance.

References

Anger K (1983) Moult cycle and morphogenesis in *Hyas araneus* larvae (Decapoda, Majidae), reared in laboratory. Helgol Meeresunters 36:285–302

Anger K (2001) The biology of decapod crustacean larvae. Crustacean Issue 14. A.A. Balkema Publishers, Swets and Zeitlinger, Lisse

Anger K (2003) Salinity as a key parameter in the larval biology of decapod crustaceans. Invertebr Reprod Dev 43(1):29–45

Anger K, Dawirs RR (1981) Influence of starvation on the larval development of *Hyas araneus* (Decapoda, Majidae). Helgol Meeresunters 34:287–311

Anger K, Dawirs RR (1982) Elemental composition (C, N, H) and energy in growing and starving larvae of *Hyas araneus* (Decapoda, Majidae). Fish Bull NOAA 80:419–433

Anger K, Jacobi CC (1985) Respiration and growth of *Hyas araneus* L. larvae (Decapoda: Majidae) from hatching to metamorphosis. J Exp Mar Biol Ecol 88:257–270

Anger K, Harms J, Püschel C, Seeger B (1989) Physiological and biochemical changes during larval development of a brachyuran crab reared under constant conditions in the laboratory. Helgol Meeresunters 43:225–244

Anger K, Spivak E, Luppi T (1998) Effects of reduced salinities on development and bioenergetics of early larval shore crab, *Carcinus maenas*. J Exp Mar Biol Ecol 220:287–304

Anger K, Thatje S, Lovrich G, Calcagno J (2003) Larval and early juvenile development of *Paralomis granulosa* reared at different temperatures: tolerance of cold and food limitation in a lithodid crab from high latitudes. Mar Ecol Prog Ser 253:243–251

Arnold KE, Findlay HS, Spicer JI, Daniels CL, Boothroyd D (2009) Effect of CO_2-related acidification on aspects of the larval development of the European lobster, *Homarus gammarus* (L.). Biogeosciences 6:1747–1754

Bechmann RK, Taban IC, Westerlund S, Godal BF, Arnberg M, Vingen S, Ingvarsdottir A, Baussant T (2011) Effects of Ocean acidification on early life stages of shrimp (*Pandalus borealis*) and mussel (*Mytilus edulis*). J Toxicol Environ Health Part A 74:424–438

Cameron JN (1978) Effects of hypercapnia on blood acid-base status, NaCl fluxes, and trans-gill potential in freshwater blue crabs, *Callinectes sapidus*. J Comp Physiol 123:137–141

Christiansen ME (1969) Crustacea Decapoda Brachyura. Marine invertebrates of Scandinavia, vol 2. Universitetsforlaget, Oslo

Dawirs RR (1983) Respiration, energy balance and development during growth and starvation of *Carcinus maenas* L. larvae (Decapoda: Portunidae). J Exp Mar Biol Ecol 69:105–128

Dissanayake A, Clough R, Spicer JI, Jones MB (2010) Effects of hypercapnia on acid–base balance and osmo/ionoregulation in prawns (Decapod: Palaemonidae). Aquat Biol 11:27–36

Drach P (1939) Mue et cycle d'intermue chez les Crustacés decapodés. Annls Institut Océanographique, Monaco 19:103–391

Dupont S, Dorey N, Stumpp M, Melzner F, Thorndyke M (2012) Long-term and trans-life-cycle effects of exposure to ocean acidification in the green sea urchin *Strongylocentrotus droebachiensis*. Mar Biol. doi:10.1007/s00227-012-1921-x

Findlay HS, Kendall MA, Spicer JI, Widdicombe S (2010) Relative influence of ocean acidification and temperature on intertidal post-larvae at the northern edge of their geographic distribution. Estuar Coast Shelf Sci 86:675–682

Hop H, Pearson T, Hegseth EN, Kovacs KM et al (2002) The marine ecosystem of Kongsfjorden, Svalbard. Polar Res 21: 167–208

Jacobi CC, Anger K (1985) Effect of temperature on respiration of larval stages of *Hyas araneus* and *H. coarctatus* (Decapoda, Majidae). Mar Ecol Prog Ser 26:181–186

Kurihara H, Ishimatsu A (2008) Effects of high CO_2 seawater on the copepod (*Acartia tsuensis*) through all life stages and subsequent generations. Mar Pollut Bull 56(6):1086–1090

Kurihara H, Shimode S, Shirayama Y (2004) Effects of raised CO_2 concentration on the egg production rate and early development of two marine copepods (*Acartia steueri* and *Acartia erythraea*). Mar Pollut Bull 49:721–727

Landry MR (1983) The development of marine calanoid copepods with comment on the isochronal rule. Limnol Oceanogr 28:614–624

Langenbuch M, Pörtner HO (2003) Energy budget of hepatocytes from Antarctic fish (*Pachycara brachycephalum* and *Lepidonotothen kempi*) as a function of ambient CO_2: pH-dependent limitations of cellular protein biosynthese? J Exp Biol 206: 3895–3903

Langenbuch M, Bock C, Leibfritz D, Pörtner HO (2006) Effects of environmental hypercapnia on animal physiology: a (31) P-NMR study of protein synthesis rates in the marine invertebrate *Sipunculus nudus*. Comp Biochem Physiol A 144:479–484

Lannig G, Eilers S, Pörtner HO, Sokolova IM, Bock C (2010) Impact of Ocean acidification on energy metabolism of Oyster, *Crassostrea gigas*—Changes in metabolic pathways and thermal response. Mar Drugs 8:2318–2339

Lewis E, Wallace DWR (1998) Program developed for CO_2 system calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, Oak Ridge, TN

Lischka S, Büdenbender J, Boxhammer T, Riebesell U (2010) Impact of ocean acidification and elevated temperatures on early juveniles of the polar shelled pteropod *Limacina helicina*: mortality, shell degradation, and shell growth. Biogeosci Discuss 7:8177–8214

Luquet CM, Ansaldo M (1997) Acid-base balance and ion regulation during emersion in estuarine intertidal crab *Chasmagnathus granulata* Dana (Decapoda Grapsidea). Comp Biochem Phys A 3:407–410

Martin S, Richier S, Pedrotti ML, Dupont S, Castejon C, Gerakis Y, Kerros ME, Oberhänsli F, Teyssié JL, Jeffree R, Gattuso JP (2011) Early development and molecular plasticity in the Mediterranean sea urchin *Paracentrotus lividus* exposed to CO_2-driven acidification. J Exp Biol 214:1357–1368

Melzner F, Gutowska MA, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M, Pörtner HO (2009) Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6:2313–2331

Michaelidis B, Ouzounis C, Paleras A, Pörtner HO (2005) Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar Ecol Prog Ser 293:109–118

Noack S (1980) Statistische Auswertung von Mess- und Versuchsdaten mit Taschenrechner und Tischcomputer. Walter de Gruyter, Berlin

Pane EF, Barry JP (2007) Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. Mar Ecol Prog Ser 334:1–9

Petersen S (1995) The embryonic development of *Hyas araneus* L. (Decapoda, Majidae): effects of temperature. Sarsia 80:193–198

Pörtner HO, Hardewig I, Sartoris FJ, van Dijk PLM (1998) Energetic aspects of cold adaptation: critical temperatures in metabolic, ionic and acid–base regulation? In: Pörtner HO, Playle R (eds) Cold ocean physiology. Cambridge University Press, Cambridge, pp 88–120

Pörtner HO, Bock C, Reipschläger A (2000) Modulation of the cost of pHi regulation during metabolic depression: a 31P-NMR study in invertebrate (*Sipunculus nudus*) isolated muscle. J Exp Biol 203:2417–2428

Pörtner HO, Dupont S, Melzner F, Storch D, Thorndyke M (2010) Studies of metabolic rate and other characters across life stages. In: Riebesell U, Fabry V, Gattuso JP (eds) Guide to best practices for ocean acidification research and data reporting. Publications Office of the European Union, Luxembourg, pp 167–180

Reipschläger A, Pörtner HO (1996) Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. J Exp Biol 199:1801–1807

Spicer JI, Eriksson SP (2003) Does the development of respiratory regulation always accompany the transition from pelagic larvae to benthic fossorial postlarvae in the Norway lobster *Nephrops norvegicus* (L.)? J Exp Mar Biol Ecol 295:219–243

Spicer JI, Raffo A, Widdicombe S (2007) Influence of CO_2-related seawater acidification on extracellular acid–base balance in the velvet swimming crab *Necora puber*. Mar Biol 151:1117–1125

Steinacher M, Joos F, Frölicher TL, Plattner G-K, Doney SC (2009) Imminent ocean acidification in the Arctic projected with the NCAR global coupled carbon cycle-climate model. Biogeosciences 6:515–533

Storch D, Santelices P, Barria J, Cabeza K, Pörtner HO, Fernández M (2009) Thermal tolerance of crustacean larvae (zoea I) in two different populations of the kelp crab *Taliepus dentatus* (Milne-Edwards). J Exp Biol 212:1371–1376

Stumpp M, Wren J, Melzner F, Thorndyke MC, Dupont S (2011) CO_2 induced seawater acidification impacts sea urchin larval development I: elevated metabolic rates decrease scope for growth and induce developmental delay. Comp Biochem Phys A 160(3): 331–340

Thomsen J, Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Mar Biol 157:2667–2676

Varley DG, Greenaway P (1992) The effect of emersion on haemolymph acid-base balance and oxygen levels in *Scylla serrata* Forskal (Brachyura: Portunidae). J Exp Mar Biol Ecol 163(1):1–12

Walther K, Anger K, Pörtner HO (2010) Effects of ocean acidification and warming on the larval development of the spider crab

Hyas araneus from different latitudes (54° vs. 79°N). Mar Ecol Prog Ser 417:159–170

Walther K, Sartoris FJ, Pörtner HO (2011) Impacts of temperature and acidification on larval calcium incorporation of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°N). Mar Biol 158:2043–2053. doi:10.1007/s00227-011-1711-x

Wheatly M, Henry RP (1992) Extracellular and intracellular acid-base regulation in crustaceans. J Exp Zool 263(2):127–142

Publication II

Pre-hatching seawater PCO_2 affects development and survival of the zoea stages of the Arctic spider crab *Hyas araneus*

Schiffer M, Harms L, Pörtner HO, Mark FC and Storch D

Marine Ecology Progress Series

(submitted)

Pre-hatching seawater P_{CO_2} affects development and survival of the zoea stages of the Arctic spider crab *Hyas araneus*

Melanie Schiffer*, Lars Harms, Hans O. Pörtner, Felix C. Mark and Daniela Storch

Department Integrative Ecophysiology, Alfred-Wegener-Institute for Polar and Marine Research,
Am Handelshafen 12, 27570 Bremerhaven, Germany

* Email: Melanie.Schiffer@awi.de

Key words: Hyas araneus, zoea, larvae, embryos, ocean acidification

Abstract

Sensitivity of marine crustaceans to anthropogenic CO_2 emissions and the associated acidification of the oceans may be less than that of other, especially lower invertebrates, however, effects on critical transition phases or carry-over effects between life stages have not comprehensively been explored. Here we report the impact of elevated seawater PCO_2 values (3100 µatm) on *Hyas araneus* during the last two weeks of their embryonic development (pre-hatching phase) and during development while in the consecutive zoea I and zoea II larval stages (post-hatching phase). We measured oxygen consumption, dry weight, developmental time and mortality in zoea I to assess changes in performance. Feeding rates and survival under starvation were investigated at different temperatures to detect differences in thermal sensitivities of zoea I and zoea II larvae depending on pre-hatch history. When embryos were pre-exposed to elevated PCO_2 during maternal care, about 60% more of them died under continued CO_2 exposure during the zoea I phase. Those larvae, which moulted into zoea II, displayed a developmental delay by about 20 days compared to larvae exposed to control PCO_2 during embryonic and zoeal phases. Elevated PCO_2 caused a reduction in zoea I dry weight and feeding rates, while survival of the starved larvae was not affected by the seawater CO_2 concentration. In conclusion, CO_2 effects on egg masses under maternal care carried over to the first larval stages of crustaceans and reduced their survival and development to levels below those previously reported in studies exclusively focussing on acute PCO_2 effects on the larval stages.

Introduction

Ongoing acidification of the world's oceans due to absorption of anthropogenic CO_2 is receiving increasing public interest. Since the early Miocene, about 24 million years ago, the atmospheric CO_2 concentrations have remained below 500 ppm CO_2 and were thus relatively stable (Pearson & Palmer 2000). Today's changes have already caused ocean pH to decline by more than 0.1 units below values characterizing pre-industrial times (Caldeira & Wickett 2003). Atmospheric CO_2 might reach values of 3000 ppm until the year 2200 (Caldeira & Wickett 2005) and cause pH to fall to 0.8 below pre-industrial vales.

Studies of the relative vulnerability of marine ectotherms to ocean acidification scenarios revealed a high inter-taxa variation in CO_2 sensitivity (Melzner et al. 2009a, Kroeker et al. 2010). Marine fish and cephalopods and to some extent, crustaceans, seem to be more tolerant to high CO_2 levels, while lower invertebrates, e.g. echinoderms and bivalves, which at the same time are more heavily calcified, appear to be more sensitive (Siikavuopio et al. 2007, Gutowska et al. 2008, Melzner et al. 2009b, Thomsen & Melzner 2010). In crustaceans as in other taxa, the tolerance to elevated CO_2 levels was found to be species dependent and linked to different ion regulation capacities (Truchot 1979, Pane & Barry 2007, Spicer et al. 2007).

An increasing number of studies in the field of ocean acidification research focus on early developmental and reproductive stages of calcifiers, which are believed to be the most vulnerable (Kurihara 2008). In crustaceans, most studies dealt with effects on post-embryonic (post-hatching) larval stages but disregarded effects of seawater P_{CO_2} on embryos pre-hatching as well as any carry-over effects on the later larval or adult stages which had been seen in molluscs or echinoderms (Parker et al. 2012, Dupont et al. 2012).

Elevated seawater P_{CO_2} of 1200 µatm CO_2 did not influence development, mortality or growth in the first two zoea stages of the European lobster *Homarus gammarus* (Arnold et al. 2009) nor zoea mortality or growth in the subtidal spider crab *Hyas araneus*. Yet, it affected the transition to the megalopa stage (Walther et al. 2010, 2011). In line with these findings, effects of elevated seawater P_{CO_2} on oxygen consumption, weight and elemental composition in developing zoea I larvae of *Hyas araneus* from an Arctic population were small, developmental duration and survival remained unaffected (Schiffer et al. 2012).

However, perturbation experiments on individual larval stages are mostly limited by the period of their development and long-term experiments are hence more difficult to carry out. The transition between larval stages may be even more critical than progression within any individual stage per se. It has therefore been criticised that short-term experiments may both over- and underestimate the real impacts of chronic exposure to high PCO_2, as there might not be enough time for acclimation or not enough time to induce effects (Dupont et al. 2012). Studies should start with the earliest and pre-hatching embryonic stages. Such efforts would take more stages into account and would extend incubation time considerably. Putative bottlenecks during development, such as the transition to the megalopa stage (Walther et al. 2010, 2011) should also be identified for a comprehensive picture.

The transition from eggs to hatching larvae might be another critical bottleneck. The eggs of almost all crustaceans are attached to the female's pleopods and subject to maternal care. After hatching, larval stages (zoea stages) are pelagic and most of them are actively swimming in the upper water column. Thus, embryos are not only exposed to the same environmental conditions as the ovigerous females but their well-being and finally, recruitment also depend on the performance capacity of the female to facilitate gas exchange of the egg masses (Fernández et al. 2000). Abiotic factors such as salinity (Giménez 2002) and temperature (Petersen 1995) experienced during the pre-hatching phase influence zoea I hatching rate, survival and development.

The first aim of this study was to investigate possible carry-over effects from CO_2 exposed embryos to the successive life history stages (zoea I and zoea II larvae) in the spider crab *Hyas araneus* collected from an Arctic population. We exposed females with late stage eggs to two different levels of seawater PCO_2 and performed a time series study on zoea I larvae hatched from these egg masses. We determined mortality and stage duration to examine larval fitness. We measured embryonic oxygen consumption (eggs) and followed the course of oxygen consumption and weight in developing zoea I larvae to estimate energy demands during continued CO_2 exposure. Heart rate and maxilliped beat rate of zoea I larvae were measured before moulting to the second stage (zoea II). We compared our present results to those of an earlier study on zoea I of Arctic *Hyas araneus* where larvae had been CO_2 exposed just after hatching but not during the preceding embryonic phase (Schiffer et al. 2012).

The second aim was to assess whether temperature sensitivities differ between zoea I or zoea II developed from control and pre-exposed egg masses. Since food availability influences the survival and development of crustacean larvae and interacts or masks effects of abiotic factors (Giménez 2002) we measured feeding rates and survival under starvation at four different temperatures in zoea I and subsequently, zoea II larvae developed from control and pre-exposed eggs. Larval feeding behaviour was then compared to observed growth rates.

Material and methods

Hyas araneus inhabits rocky, sandy and muddy bottoms on continental shelves, and has a wide distribution from the temperate southern North Sea to sub-Arctic waters (Christiansen 1969). It is a model organism to study the effects of elevated seawater P_{CO_2} on successive life-history stages (Walther et al. 2010, Walther et al. 2011, Schiffer et al. 2012). Females carry egg masses for approximately 2 years (Petersen 1995). Zoea I larvae hatch from the eggs and are released into the water column. After going through two zoeal and one megalopa stage juveniles settle in the adult habitat.

Egg and larval collection and maintenance

Ovigerous females of *Hyas araneus* were collected in Kongsfjorden (Ny Alesund, Svalbard 78°55 N, 11°56 E, Arctic population) by scientific divers during spring 2010 at 8 m depth and a water temperature of 0°C. They were transferred to the Alfred Wegener Institute in Bremerhaven and maintained in flow-through aquaria at 4°C during summer and at 0°C during early winter to avoid early hatching at a salinity of 31 psu. In January 2011 females with late stage eggs were placed individually in 2 l flow-through aquaria within recirculating CO_2 incubation systems (volume 1m^3 seawater each) at 4,5 ± 0,1°C to induce hatching. Seawater CO_2 manipulation was achieved by injecting the feeder tank and the header tank with a defined air /CO_2 mixture using a mass flow controller (HTK 6 channel, HTK Hamburg GmbH, Germany). Aquaria were directly provided with seawater from the header tank. Egg carrying females were held at two different CO_2 concentrations of 350 µatm (controls) and 3100 µatm CO_2 (high CO_2 treatment), respectively. Hatching started approximately two weeks after the females had been transferred to incubation systems.

Experiments were conducted with zoea I larvae that had hatched within 24 h. Hatched larvae from three females were pooled and groups of 30 individuals were transferred into 0.5 l culture vessels, with seawater CO_2 concentrations maintained. Larvae were kept at a

constant temperature of 4.4 ± 0.4°C. Seawater was provided from reservoir tanks (60l) injected with a defined air /CO_2 mixture using a mass flow controller (HTK 6 channel, HTK Hamburg GmbH, Germany). Water in culture vessels and food (freshly hatched *Artemia* sp., Sanders Brine Shrimp Company, Ogden, Utah, USA) were changed daily and dead larvae and moults were removed. Zoea II that had moulted on the same day were pooled and transferred to a new culture vessel. Water physicochemistry was monitored weekly by determining pH and collecting water samples for the analysis of dissolved inorganic carbon (DIC). Water P_{CO_2} was calculated from DIC and pH_{NBS} using the program CO_2 SYS (Lewis & Wallace 1998) (Tab. 1).

Mortality and developmental time

22 culture vessels containing about 30 larvae each were used for investigating the effect of control (11 vessels) or elevated CO_2 (11 vessels) on mortality and duration of the larval stage. Mortality and moulting (number of zoea II) were recorded on a daily basis until all larvae were either dead or moulted into zoea II. Dead larvae and zoea II were removed. Survival curves for larvae were generated and medium survival was determined using GraphPad Prism 4. Differences in survival curves between control and high CO_2 exposures were tested by a Logrank test. Mean developmental times for larvae under control and elevated CO_2 levels were determined from means of the eleven replicates using GraphPad Prism 4.

As high CO_2 concentrations frequently affect larval developmental time, the *virtual age* of zoea I larvae were calculated after Pörtner et al. (2010) to detect day-specific differences in larval dry weight. *Virtual age* was calculated as "real age (days post hatching) / effect size" (Tab. 2) where "effect size" is defined as the ratio "zoea I stage duration at 350 µatm CO_2 / zoea I stage duration at 3100 µatm CO_2".

Oxygen consumption and dry weight

Oxygen consumption rates of eggs and individual zoea I larvae were measured in closed, double-walled respiration chambers (OXY041 A, Collotec Meßtechnik GmbH, Niddatal, Germany) perfused by thermostatted water to maintain control temperature at 4°C. Oxygen saturation was recorded by oxygen micro-optodes (needle-type NTH-PSt1-L5-TF-NS*46/0,80-YOP, fiber-optic microsensor, flat broken tip, diameter: 140 µm, PreSens GmbH, Regensburg, Germany), connected to a Microx TX3 (PreSens GmbH, Regensburg, Germany).

Oxygen consumption rates were measured in egg batches from 8 individual females after one week of incubation. Approximately 20 eggs were carefully removed from each brooding female and placed on a fine grid in the respiration chamber. A magnetic stirrer was placed beneath the grid and the water in the respiration chamber was gently mixed to prevent oxygen stratification. The plunger of the chamber lid was inserted and the volume of the chamber was reduced to 250 µl. Respiration measurements were stopped when the oxygen saturation of the chamber water had decreased to about 80% air saturation. Before and after each measurement, blanks were run to consider bacterial oxygen consumption.

Hatched larvae were carefully transferred from the culture vessel into the respiration chamber, which contained seawater of the corresponding CO_2 condition. Afterwards the plunger of the chamber lid was inserted and the volume of the chamber was reduced to 150 µl. The needle of the micro-sensor was inserted into the chamber through a hole in the lid and the sensitive tip of the optode was placed in the middle of the chamber. The almost constant swimming of the larvae caused mixing of the water in the chamber. Respiration measurements were stopped after thirty minutes or when the oxygen saturation of the chamber water had decreased to about 80%. Before or after each measurement, blanks were run to account for bacterial oxygen consumption. Larval oxygen consumption was expressed as $\mu gO_2/mgDW*h^{-1}$ ±SE to allow for treatment-specific differences in larval dry weight.

After oxygen consumption measurements, eggs/larvae were removed from the chamber and briefly rinsed with deionized water. Excessive water was removed using a paper towel. Larvae/eggs were killed by snap-freezing and stored at -20°C in pre-weighed tin cartridges for dry weight determination by freeze-drying to constant weight determined on a high precision balance (Mettler Toledo AG, Greifensee, CH-8606, CH). Larval dry weight is given as µg ± SE per individual. At all sampling days eight zoea I larvae and egg masses, respectively, from each CO_2 treatment were used to measure oxygen consumption. The same eight individuals were used for dry weight analyses. Oxygen consumption rates and weight were plotted against real age (days post hatching) and virtual age (dph/effect size) to compare their change over time courses in regard to actual developmental day as well as compensated for developmental delay in high CO_2 larvae.

Heart rate and maxilliped activity

In addition to oxygen consumption, heart rate and maxilliped activity were determined in

five zoea I larvae on day 50 post-hatching by using a digital camera (AxioCam MRm, Carl Zeiss, Mikroimaging GmbH, Göttingen, Germany) mounted onto a microscope (Axio Observer A1, Carl Zeiss). Larvae were measured under the microscope in a custom-built temperature-controlled flow-through micro-chamber (AWI workshop) with a flow rate of 5 ml/min to avoid a decrease in oxygen concentration due to larval respiration in the closed chamber. Temperature controlled seawater (4°C temperature, 32 PSU salinity) was provided from a reservoir vessel placed in the thermostat water bath and was pumped through the chamber. Before closing the chamber individual larvae were positioned in the centre of the micro-chamber by gluing the carapace to a thin glass spine, which itself was attached to a glass table. Then the chamber was closed and water flow through the chamber was started. Larvae were left for 1 h to recover from handling stress and were videotaped for 1 min periods. The video sequence was analysed for heartbeat and maxilliped activity, respectively, by counting the beats min^{-1}. The beating heart can easily be seen through the transparent carapace. Heart rate and maxilliped beat rate were calculated for each larva as the mean number of beats min^{-1} ±SE from three 10s intervals.

Feeding rate

Feeding rates were measured on day 8 post-hatching in zoea I and on day 8 post-moulting in zoea II larvae. To determine feeding rates, larvae were held individually in closed vials containing 10 ml of seawater at constant salinity of 32 PSU. One day prior to experiments six vials per CO_2 treatment were placed at four different temperatures (3°C, 9°C, 15°C and 21°C, 48 vials in total for zoea I and 48 vials for zoea II) in a temperature-controlled table providing a stable temperature gradient (custom-made by AWI workshop). Larvae were starved for two days at rearing temperature and then transferred to experimental temperatures for one day prior to feeding experiments. On day four, *Artemia* sp. (Sanders Brine Shrimp Company, Ogden, Utah, USA) were added to a density of 10 animals/ml^{-1} per vial. After 24 h the larvae were carefully removed from the incubation vial leaving Artemia specimens behind. The remaining *Artemia* individuals were counted under a microscope and larvae were killed by snap-freezing and stored at -20°C. Feeding rate is given as number of *Artemia*/$Ind^{-1}d^{-1}$ ±SE.

Survival under starvation

Larvae were fed until day 8 post-hatching in zoea I or until day 8 post-moulting in zoea II as described above. Subsequently, larvae were transferred individually into closed vials containing 10ml seawater of a constant salinity of 32 PSU. Individual rearing was necessary to avoid cannibalism during the starvation experiment. One day prior to

experiments six vials per CO_2 treatment were exposed to four different temperatures (3°C, 9°C, 15°C and 21°C) in a temperature-controlled table. Seawater was provided from reservoir tanks (4,5 ± 0,1°C) that were injected with a defined air /CO_2 mixture. Water in the experimental vials was changed daily and checked for dead larvae. Larvae were considered dead when no heartbeat could be detected under a microscope.

Statistical analyses

Results were analysed using GraphPad Prism 4. All data were checked for outliers by use of Nalimov's test (Noack 1980). A two-way ANOVA was used to investigate the effects of CO_2 concentration and day of development on larval oxygen consumption and dry weight, as well as the effects of CO_2 concentration and temperature on survival under starvation, feeding rates, oxygen consumption and heart rates. Bonferroni tests were used for *a posteriori* analyses. When a disordinal interaction between factors was detected, a one-way ANOVA was run additionally for each CO_2 concentration to detect differences among days of development/temperatures. Tukey's multiple comparison tests were used for *a posteriori* analyses. An unpaired t-test was conducted to analyse the effect of CO_2 on egg respiration, zoea I development time and heart beat rate, maxilliped beat rate and oxygen consumption in 50 day old zoea I larvae. A multiple linear regression was calculated with dry weight and respiration as dependent variable using SigmaPlot (Version 12, Systat Software, Inc., San Jose, California).

Results

Mortality and developmental time (zoea I larvae)

Survival curves of larvae reared at control versus elevated P_{CO_2} values were significantly different (Logrank test $p<0.0001$) (Fig. 1). Larvae from females pre-exposed to high CO_2 had a medium survival (LD 50) of 74 days, while no medium survival could be determined for control larvae as percent survival at the end of the study exceeded 50%. 90.1% of larvae reared at control CO_2 levels and 26.2% kept at high CO_2 levels, respectively, survived the first zoea stage and moulted into zoea II (Fig. 1). Seawater CO_2 concentration had a significant effect on the duration of the first zoea stage (unpaired t-test $p<0.0001$). Stage duration until moulting into zoea II increased from 47.7±0.8 days under control condition to 68.1±6.2 days at high CO_2 levels (Fig. 1).

Oxygen consumption (eggs and zoea I larvae)

There was no effect of seawater CO_2 concentration on mean egg respiration rates (unpaired t-test p=0.1309), which was 0.33±0.03 SE μgO_2/mg DW*h^{-1} in eggs from females under control P_{CO_2} and 0.44±0.05 μgO_2/mg DW*h^{-1} in eggs from the high CO_2 treatment (Fig. 2A).

There was a significant but disordinal interaction in the two-way ANOVA between day of development and CO_2 concentration in zoea I larvae (Tab. 3), which renders an interpretation of both main factors (CO_2 and day of development) impossible. Neither seawater CO_2 concentration had an effect on larval respiration, nor day of development (p>0.5). The interaction was significant because larvae reared at high CO_2 showed higher respiration rates on day 2 after hatching and lower rates during further development in comparison with control larvae. When data of day 2 were excluded from the two-way ANOVA, seawater CO_2 concentration had a significant effect on larval respiration rates (ANOVA p<0.01, F=8.096) while day of development showed no effect. There was a significant but disordinal interaction in the two-way ANOVA between day of development and CO_2 concentration when egg respiration was included in the two-way ANOVA, which renders a global interpretation of both main factors (CO_2 and day of development) impossible.

Respiration rates of zoea I larvae reared under control conditions ranged from 0.80 on day 2 to a maximum of 1.44 μgO_2/mgDW*h^{-1} on day 36, but did not change significantly with proceeding development (one-way ANOVA p<0.05, F=0.9124) (Fig. 2A). Respiration rates changed slightly with proceeding development when egg respiration was included in the one-way ANOVA (p=0.0088, F=2.924) and increased significantly from day 5 pre-hatching to day 36 post-hatching (p<0.001). There was a significant effect of age on overall respiration rates in zoea I larvae incubated at high CO_2 levels (one-way ANOVA p<0.05, F=3.506). Application of an *a posteriori* Tukey's test showed a significant decrease in respiration rates from 1.57 on day 2 to 0.41 μgO_2/mg DW*h^{-1} on day 8 and constant rates between day 8 and day 48 (p>0.05). When egg respiration was included in the one-way ANOVA an *a posteriori* Tukey's test showed a significant increase between day 5 pre-hatching and day 2 post-hatching (p<0.001).

The zoea I stage duration was 1.37 times longer in the CO_2 treatment compared to the control treatment (Tab.2). When related to virtual age a multiple regression analysis indicated a dependence of oxygen consumption rates on seawater CO_2 concentration (p<0.001) (Fig. 2B) but not on time (virtual age)(p<0.884). Data for day 2 were excluded

from this analysis to improve the curve fit.

Dry weight (zoea I larvae)

There was a significant ordinal interaction in the two-way ANOVA between day of development and CO_2 concentration (Tab. 3), allowing for an interpretation of both main factors (CO_2 and temperature) with respect to effects on dry weight. Larval dry weight increased over time in both treatments (Fig. 3A) from 0.0926 and 0.0946 on day 2 to 0.1778 and 0.1628 µg on day 48 under control conditions and under high CO_2 conditions, respectively. In zoea I larvae reared at control CO_2 levels, dry weight increased between day 2 and day 32 (p<0.001) and remained constant between day 32 and day 48 (p>0.05) (Fig. 3A). There was no difference between dry weights of larvae from the incubation at 3100 µatm CO_2 after day 2 and day 16 (p>0.05). Between day 16 and 48 dry weight increased significantly (p<0.001). Starting with the same dry weight as control larvae on day 2, larvae reared at high CO_2 showed a reduced dry weight during their development (p<0.05) but finally dry weight equalled that of control larvae on day 48 (Fig. 3A). When related to virtual age the linear regression in dry weight in larvae from both CO_2 concentrations was dependent on both factors day (p<0.001) and seawater CO_2 concentration (p<0.001) with lower dry weight in larvae from the high CO_2 treatment (Fig. 3B).

Oxygen consumption, heart rate and maxilliped activity (zoea I larvae)

Oxygen consumption of 50 days old zoea I larvae did not differ between CO_2 treatments (p>0.05), while heart beat rate was significantly reduced at elevated P_{CO_2} (p=0.0061) (Fig. 4). Heart beat rate was 97±5 beats/min in larvae reared at control CO_2 and 69±3 beats/min in larvae reared at high CO_2 levels (Fig. 4). Maxilliped activity decreased significantly with elevated seawater P_{CO_2} from 85±15 beats/min in control larvae to 33±7 beats/min in larvae under high CO_2 levels (p=0.0394) (Fig. 4).

Feeding rates (zoea I and zoea II larvae)

Feeding rates of zoea I larvae (8 days post-hatching) were significantly affected by both CO_2 concentration and temperature (Tab. 4). Under control conditions, feeding rates of zoea I larvae increased significantly from 17±2 *Artemia* at 3°C to 33±4 *Artemia*/Ind^{-1}*d^{-1} at 9°C (p<0.01), remained constant between 9°C and 15°C and decreased significantly at 21°C to 17±2 *Artemia*/Ind^{-1}*d^{-1} (Fig. 5A). Zoea I larvae reared under elevated CO_2 levels displayed no significant changes in feeding rate with temperature, ranging from 8±2 at 3°C to 17±2 *Artemia*/Ind^{-1}*d^{-1} at 15°C (p>0.05) and were always below those of the control

larvae. While seawater P_{CO_2} had no effect on feeding rate at 3°C and 21°C, feeding rates at 9°C and 15°C were significantly reduced in zoea I larvae from the high CO_2 treatment in comparison to control zoea I larvae (9°C: $p<0.001$; 15°C: $p<0.05$).

A similar trend could be found in zoea II larvae (8 days post-moulting). There was a significant ordinal interaction in the two-way ANOVA between temperature and CO_2 concentration (Tab. 4). Feeding rates of control larvae at 21°C were excluded from the ANOVA as no data were available from larvae raised at high CO_2 levels at 21°C due to 100% mortality. Both CO_2 concentration and temperature had a significant effect on zoea II feeding rates (Tab. 4). Feeding rates increased significantly with increasing temperature from 19±1 Artemia at 3°C to 70±3 *Artemia*/Ind^{-1}*d^{-1} at 15°C in larvae reared under control conditions ($p<0.01$) (Fig. 5B). In zoea II larvae raised at elevated P_{CO_2} feeding rates increased significantly between 3°C and 15°C ($p<0.01$). There were no differences in feeding rates between 3°C and 9°C or 9°C and 15°C, respectively. At 9°C and 15°C, feeding rates of larvae reared under high P_{CO_2} were significantly reduced in comparison to control zoea II larvae (9°C: $p<0.001$; 15°C: $p<0.001$).

Survival under starvation (zoea I and zoea II larvae)

Temperature had a significant effect on the survival time of starved zoea I larvae (experiment started 8 days post-hatching, Tab. 2) with decreasing survival at increasing temperature (Fig. 6A). The mean survival time of starved larvae incubated under control conditions decreased significantly from 26±5 days at 3°C to 19±2 days at 9°C and 7±1 days at 15°C ($p<0.01$). There was no difference in larval survival between 15°C and 21°C. Survival of starved zoea I larvae reared at high CO_2 levels decreased significantly between 3°C and 9°C ($p<0.001$) and remained constant between 9°C and 21°C. Seawater CO_2 concentration had no effect on the survival of starving zoea I larvae (Tab. 4).

Survival of zoea II *Hyas araneus* larvae (experiment started 8 days post-moulting) was also significantly affected by temperature (Tab. 2, Fig. 6B). Larvae from both CO_2 treatments showed a significantly decreased survival at 21°C compared to survival at 3°C (350 µatm CO_2: $p<0.05$; 3100 µatm CO_2: $p<0.001$) (Fig. 6B). Survival time under starvation decreased from 34±4 and 30±6 days at 3°C to 17±0.5 and 5±1 days at 21°C under control and high CO_2 conditions, respectively. Seawater CO_2 concentration had no effect on survival of starved zoea II larvae (Tab. 4).

Discussion

Our data collected under elevated CO_2 levels demonstrate the need to focus on early ontogenetic development as a whole rather than on selected life stages. Sensitivities of different life stages to CO_2 are co-defined by their preceding life stages or the transition from one to the next stage.

The early embryonic development of (sub-) Arctic *Hyas araneus* is unusual, as the total time span of approximately 2 years between the spawning of eggs and the hatching of larvae is exceptionally long (Petersen 1995). The present study revealed that exposure of females with late stage eggs to high seawater P_{CO_2} values, during a comparatively short period of two weeks, already had fundamental consequences for larval performance indicated by high mortality and prolonged developmental time in the first larval stage (zoea I). In contrast, elevated seawater CO_2 concentrations had no effect on zoea I mortality and developmental duration of (sub-) Arctic *Hyas araneus* when CO_2 exposure started post-hatching (Schiffer et al. 2012). This conclusion is further supported by studies of Walther et al. (2010, 2011) and Arnold et al. (2009), who found no effect of elevated seawater P_{CO_2} on mortality or growth of the first zoea stages in the spider crab *Hyas araneus* and the European lobster *Homarus gammarus*. In *Hyas araneus*, the megalopa stage was more sensitive than the preceding stages (Walther et al. 2010), also involving a potential carry-over effect. These findings suggest that high seawater P_{CO_2} negatively affects either pre-hatching embryonic development or key developmental events - such as the remodelling occurring during hatching – adversely affecting the larvae and resulting in elevated mortalities.

Pre-hatching embryonic development could be affected due to higher CO_2 exposure of the embryos or due to modified behaviour of the female. Like in many marine decapod crustaceans, *Hyas araneus* eggs are attached to the female's pleopods and provided with fresh seawater by the female's behaviour, sustaining oxygen availability to these compact egg masses (Fernández et al. 2000, Baeza & Fernández 2002). Embryonic development is oxygen limited (Fernández et al. 2003). Oxygen limitation would be exacerbated by enhanced embryonic oxygen demand (non-significant in the present study, after 2 weeks of pre-hatching CO_2 exposure) or a limiting role for female ventilation, especially in the center of the egg mass. Progressive oxygen limitation goes hand in hand with CO_2 accumulation from respiration and thereby exacerbates exposure to elevated P_{CO_2}. Increased embryonic respiration was found during long-term exposure (14 weeks) at elevated seawater P_{CO_2} and led to enhanced ventilation activity in female *Hyas araneus*

(Schiffer et al. in prep).

Elevated respiration rates at increased seawater P_{CO_2} in zoea I larvae two days post-hatching indicate that high seawater CO_2 levels might also affect the hatching process. The first instar of *Hyas araneus* is a prezoea, which lasts between a few minutes to a few hours before moulting into the first zoea stage (Anger 1983). Hatching and moulting are characterised by a pronounced uptake of water, while the percent water content is decreasing with proceeding development (Anger & Dawirs 1982). During this transitional phase of remodelling, larvae may be especially sensitive to environmental stressors or disturbances in acid-base status. High oxygen consumption rates in zoea I larvae two days post-hatching indicate an uncompensated energetic cost associated with maintenance. Dawirs (1983) estimated that respiration comprised 16%, while growth comprised 10% and egestion/excretion 71% of the total energy assimilated by food intake in zoea I larvae of *Carcinus maenas*. Any increase in the cost of maintenance, such as due to enhanced ion and acid-base regulation, would reduce the amount of energy available for other processes like growth. The increase in dry weight was actually less during the first two weeks post-hatching in *Hyas araneus* larvae exposed to elevated P_{CO_2}.

There is a growing body of evidence that embryonic exposure to elevated P_{CO_2} elicits behavioural (and physiological) changes in hatched larvae of marine ectotherms. At a seawater P_{CO_2} of 1100 µatm CO_2 during embryogenesis embryos of the intertidal snail *Littorina obtusata* changed their behaviour, by spending more time stationary instead of crawling, as well as displaying slower rotation rates (Ellis et al. 2009). Altered behaviour could also be found in larvae of coral reef fish, when eggs and larvae were exposed to elevated seawater P_{CO_2} (Munday et al. 2009, Dixson et al. 2010). In the present study, *Hyas araneus* zoea I and zoea II larvae experiencing elevated seawater P_{CO_2} during their embryonic phase, also displayed altered behaviour. This was reflected in reduced feeding rates in early zoea I and zoea II larvae and reduced maxilliped beat rates in older zoea I larvae. Although not significant, mean feeding rates of larvae were lower at elevated seawater P_{CO_2} at 3°C rearing temperature. At elevated P_{CO_2} lower feeding rates were accompanied by lower oxygen consumption rates indicating lower energy demands, possibly due to pH induced metabolic depression, which then involves lower maxilliped activity for oxygen supply. Less energy might also be used for swimming and feeding. Lowered energy demand is also indicated by lower heart rates in 50-day old, P_{CO_2} treated larvae.

The differences in feeding rates became more pronounced at 9°C and 15°C. At 21°C feeding rates of control larvae decreased and no differences were found between larvae from the two CO_2 treatments. Within the thermal window increasing oxygen consumption rates with increasing seawater temperature indicate higher metabolic costs for maintenance in *Hyas araneus* zoea larvae (Jacobi & Anger 1985). Higher metabolic costs were met up to a certain temperature by increased feeding rates in control larvae, but not in larvae kept at high PCO_2 levels.

Zoea of Brachyuran crabs are carnivorous (Le Vay et al. 2001) and catch live prey by using the telson to scoop up the prey and hold it from below (Gonor & Gonor 1973). Good swimming abilities are a crucial prerequisite for successful foraging. However, elevated temperatures as used in feeding experiments can influence animal performance, which depends on aerobic scope (Pörtner & Farrell 2008). Elevated seawater PCO_2 might limit the scope for aerobic performance at elevated temperature in larvae reared at high seawater CO_2 and might prohibit an increase in activity levels and thereby, appropriate feeding rates. A reduction of aerobic scope at thermal extremes has already been shown by Walther et al. (2009) for *Hyas araneus* adults exposed to elevated seawater PCO_2.

Larval dry weight is a suitable indicator for growth and feeding in crustacean larvae and reflects the level of food supply. To our knowledge this is the first study showing reduced growth under elevated seawater PCO_2 levels in the first larval stage of decapod crustaceans. Especially during the first two weeks of development the dry weight increment was less in *Hyas araneus* zoea I exposed to elevated PCO_2 (3% compared to 50% between day 2 and day 16). A difference in dry weight increments between control and CO_2 treated larvae has so far only been found in late developmental stages (Arnold et al. 2009, Walther et al. 2010). A shift in energy budget might occur with more energy spent on ion and acid-base regulation and less on growth. As a dilemma, the behavioural depression causing lower feeding rates reduces energy availability and may thereby exacerbate the reduction in growth.

Different larval feeding strategies can be found in crustaceans. While larval development of some crustacean species at high latitudes comprises a (non-feeding) phase supported by yolk consumption (Anger et al. 2003), Arctic *Hyas araneus* larvae depend on immediate food supply for a successful development. Earlier studies indicate the crucial importance of sufficient food supply for development and survival of crustacean larvae (Anger & Dawirs 1981, Dawirs 1983). The influence of starvation on larval survival and

development became particularly evident when larvae were starved at the beginning of their development (Anger & Dawirs 1981). Zoea I mortality of *Hyas araneus* from the North sea was over 90% when feeding was prevented on the second day post hatching. However, after four days or more of feeding, starvation did not influence survival to the second stage. Feeding conditions also affected the duration of larval development in the zoea I. Development was almost twice as long in larvae starved during the first six days post hatching than under normal feeding conditions (Anger & Dawirs 1981). Thus, we assume that the high mortality and prolonged development found in *Hyas araneus* zoea I larvae reared at elevated PCO_2 can partly be attributed to reduced feeding rates. This assumption is further supported by the fact that the seawater PCO_2 did not affect survival time of *Hyas araneus* zoea I and zoea II once starved. Then temperature but not CO_2 concentration determines survival time. Hence, sustained feeding would support larval survival under elevated seawater CO_2 concentrations.

Reduced feeding and developmental delay at elevated seawater PCO_2 has also been found in a previous study on sea urchin larvae (Stumpp et al. 2011). Larvae of similar size had a comparable feeding efficiency indicating that reduced feeding paralleled the developmental delay. In the present study, feeding rates in *Hyas araneus* zoea I larvae were solely investigated at day 8 post-hatching, when larvae reared at high PCO_2 had reached a *virtual age* of 6 days. Hence, reduced feeding rates and a lower increase in dry weight might cause the developmental delay in larvae reared at elevated PCO_2. We hypothesize that elevated PCO_2 causes developmental delay through reduced feeding or vice versa mirroring a coordinated reduction of both.

Conclusion

We investigated how the exposure of ovigerous females and their embryos to a late ocean acidification scenario affects the subsequent larval stages of the spider *crab Hyas araneus*. We demonstrated that after such pre-exposure the survival and development of the first zoea I stage is highly affected by elevated seawater PCO_2. In contrast, there was no effect of seawater PCO_2 on the survival and development of zoea I acutely exposed to different seawater PCO_2 values (Schiffer et al. 2012). Our study emphasizes the need for comprehensive studies of the whole life cycle of a species for the development of the full effect. Carry-over effects between life stages and/or CO_2 induced disturbances of the transition phases from one to the next stage have the potential to severely impact species survival in a high CO_2 world.

Acknowledgements

Financial support was provided by Federal Ministry of Education and Research (BMBF), within phase I of the BIOACID research programme (FKZ 03F0608B, subproject 2.2.1 PI D. Storch). We thank the scientific divers of the Alfred Wegener Institute for animal collection and L. Preis, P. Augustin, M. Zhu and P. Schulze for technical assistance.

References

Anger K (1983) Moult cycle and morphogenesis in *Hyas araneus* larvae (Decapoda, Majidae), reared in laboratory. Helgol Meeresunters 36: 285-302

Anger K, Dawirs RR (1981) Influence of starvation on the larval development of *Hyas araneus* (Decapoda, Majidae). Helgol Meeresunters 34: 287-311

Anger K, Dawirs RR (1982) Elemental composition (C,N,H) and energy in growing and starving larvae of *Hyas araneus* (Decapoda, Majidae). Fish Bull NOAA 80: 419- 433

Anger K, Thatje S, Lovrich G, Calcagno J (2003) Larval and early juvenile development of *Paralomis granulosa* reared at different temperatures: tolerance of cold and food limitation in a lithodid crab from high latitudes. Mar Ecol Prog Ser 253: 243–251

Arnold KE, Findlay HS, Spicer JI, Daniels CL, Boothroyd D (2009) Effect of CO_2-related acidification on aspects of the larval development of the European lobster, *Homarus gammarus* (L.). Biogeosciences 6: 1747-1754

Baeza JA, Fernández M (2002) Active brood care in *Cancer setosus* (Crustacea: Decapoda): the relationship between female behaviour, embryo oxygen consumption and the cost of brooding. Funct Ecol 16: 241-251

Caldeira K, Wickett ME (2003) Anthropogenic carbon and ocean pH. Nature 425: 365

Caldeira K, Wickett ME (2005) Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J Geophys Res 110, C09S04, doi:10.1029/2004JC002671

Christiansen ME (ed) (1969) Crustacea Decapoda Brachyura. In: Marine invertebrates of Scandinavia, Vol. 2, Universitetsforlaget, Oslo

Dawirs RR (1983) Respiration, energy balance and development during growth and starvation of *Carcinus maenas* L. larvae (Decapoda: Portunidae). J Exp Mar Biol Ecol 69: 105-128

Dixson DL, Munday PL, Jones GP (2010) While Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. Ecol Lett 13: 68–75

Dupont S, Dorey N, Stumpp M, Melzner F, Thorndyke M (2012) Long-term and trans-life-cycle effects of exposure to ocean acidification in the green sea urchin *Strongylocentrotus droebachiensis*. Mar Biol DOI 10.1007/s00227-012-1921-x

Ellis RP, Bersey J, Rundle SD, Hall-Spencer JM, Spicer JI (2009) Subtle but significant effects of CO_2 acidified seawater on embryos of the intertidal snail, *Littorina obtusata*. Aquat Biol 5: 41–48

Fernández M, Bock C, Pörtner HO (2000) The cost of being a caring mother: the ignored factor in the reproduction of marine invertebrates. Ecol Lett 3: 487-494

Fernández M, Ruiz-Tagle N, Cifuentes S, Pörtner HO, Arntz W (2003) Oxygen-dependent asynchrony of embryonic development in embryo masses of brachyuran crabs. Mar Biol 142: 559–565

Giménez L (2002) Effects of prehatching salinity and initial larval biomass on survival and duration of development in the zoea 1 of the estuarine crab, *Chasmagnathus granulata*, under nutritional stress. J Exp Mar Biol Ecol 70 (1): 93-110

Gonor SL, Gonor JJ (1973) Feeding, cleaning and swimming behaviour in larval stages of Porcellanid crabs (Crustacea: Anomura). Fish B-NOAA 71 (1): 225-234

Gutowska MA, Pörtner HO, Melzner F (2008) Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater. Mar Ecol Prog Ser 373: 303–309

Jacobi CC, Anger K (1985) Effect of temperature on respiration of larval stages of *Hyas araneus* and *H. coarctatus* (Decapoda, Majidae). Mar Ecol Prog Ser 26: 181-186

Kroeker KJ, Kordas RL, Crim RN, Singh GG (2010) Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol Lett 13: 1419–1434

Kurihara H (2008) Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. Mar Ecol Prog Ser 373: 275–284

Lewis E, Wallace DWR (1998) Program developed for CO_2 system calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, Oak Ridge, TN

Le Vay L, Jonesa DA, Puello-Cruzb AC, Sanghab RS, Ngamphongsaic C (2001) Digestion in relation to feeding strategies exhibited by crustacean larvae. Comp Biochem Physiol A 128: 623-630

Melzner F, Gutowska MA, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M, Pörtner HO (2009a) Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6: 2313–2331

Melzner F, Göbel S, Langenbuch M, Gutowska MA, Pörtner HO, Lucassen M (2009b) Swimming performance in Atlantic Cod (*Gadus morhua*) following long-term (4–12 months) acclimation to elevated seawater PCO_2. Aquat Toxicol 92: 30–37

Munday PL, Dixson DL, Donelson JM, Jones GP, Pratchett MS, Devitsina GV, Døving KB (2009) Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *PNAS* 106 (6): 1848-1852

Noack S (1980) Statistische Auswertung von Mess- und Versuchsdaten mit Taschenrechner und Tischcomputer. Berlin: Walter de Gruyter

Pane EF, Barry JP (2007) Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. Mar Ecol Prog Ser 334: 1-9

Parker LM, Ross PM, O'Connor WA, Borysko L, Raftos DA, Pörtner (2012) Adult exposure influences offspring response to ocean acidification in oysters. Glob Change Biol 18: 82–92

Pearson PN, Palmer MR (2000) Atmospheric carbon dioxide concentrations over the past 60 million years. Nature 406: 695-699

Petersen S (1995) The embryonic development of *Hyas araneus* L. (Decapoda, majidae): Effects of temperature. Sarsia 80 (3): 193-198

Pörtner HO, Farrell AP (2008) Physiology and climate change. Science 31 (322): 690-692

Pörtner HO, Dupont S, Melzner F, Storch D, Thorndyke M (2010) Studies of metabolic rate and other characters across life stages. In: Riebesell U, Fabry VJ, Hansson L and Gattuso J-P (eds) Guide to best practices for ocean acidification research and data reporting. Luxembourg: Publications Office of the European Union, p 167-180

Siikavuopio S, Mortensen A, Dale T, Foss A (2007) Effects of carbon dioxide exposure on feed intake and gonad growth in green sea urchin, *Strongylocentrotus droebachiensis*. Aquaculture 266, 1-4: 97-101

Schiffer M, Harms L, Pörtner HO, Lucassen M, Mark FC, Storch D (2012) Tolerance of *Hyas araneus* zoea I larvae to elevated seawater PCO_2 despite elevated metabolic costs. Mar Biol DOI 10.1007/s00227-012-2036-0

Spicer JI, Eriksson SP (2003) Does the development of respiratory regulation always accompany the transition from pelagic larvae to benthic fossorial postlarvae in the Norway lobster *Nephrops norvegicus* (L.)? J Exp Mar Biol Ecol 295: 219-243

Truchot JP (1979) Mechanisms of the compensation of blood respiratory acid-base disturbances in the shore crab, *Carcinus maenas* (L.). J Exp Zool 210 (3): 407–416

Thomsen J, Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Mar Biol 157: 2667–2676

Walther K, Sartoris FJ, Bock C, Pörtner HO (2009) Impact of anthropogenic ocean acidification on thermal tolerance of the spider crab *Hyas araneus*. Biogeosciences Discuss 6: 2837–2861

Walther K, Anger K, Pörtner HO (2010) Effects of ocean acidification and warming on the

larval development of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°N). Mar Ecol Prog Ser 417: 159-170

Walther K, Sartoris FJ, Pörtner HO (2011) Impacts of temperature and acidification on larval calcium incorporation of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°N). Mar Biol 158: 2043-2053

Tables

Table 1 Seawater parameters measured during incubation. Values are given in mean ± SD. N = 4 NBS: National Bureau of Standards; DIC: dissolved inorganic carbon; P_{CO_2}: partial pressure of CO_2.

Incubation	Temperature (C°)	pH_{NBS}	DIC (µmol/kg)	P_{CO_2} (µatm)	Salinity (PSU)
Control	4.5±0.5	8.13±0.05	2308±83	349±57	31.2±0.9
CO_2 3100	4.4±0.2	7.20±0.02	2492±73	3103±146	31.2±0.9

Table 2 Zoea I stage duration (days) and effect size (relative to zoea I reared at 350 µatm) used for calculating the virtual age (virtual age (d) = real age (d) / effect size). Effect size is defined as the ratio "zoea I stage duration at 350 µatm CO_2 / zoea I stage duration at 3100 µatm CO_2"

P_{CO_2}	**Zoea I stage duration (days)**	**Effect size**
350 µatm	51.95	1
3100 µatm	71.30	0.72

Table 3 Results of 2-way ANOVAs conducted to investigate effects of CO_2 and larval age oxygen consumption and dry weight of *H. araneus* zoea I larvae, respectively.

Response variable	**CO_2 effect**			**Day of development**			**Interaction**		
	F	**d.f.**	**P**	**F**	**d.f.**	**P**	**F**	**d.f.**	**P**
Oxygen consumption	2.662	1	0.1060	1.720	7	0.1130	2.583	7	**0.0174**
Dry weight	91.02	1	**<0.0001**	53.74	7	**<0.0001**	2.717	7	**0.0132**

Table 4 Results of 2-way ANOVAs conducted to investigate effects of CO_2 and temperature on feeding rate and survival under starvation of *H. araneus* zoea I and zoea II larvae, respectively.

Response variable	CO_2 effect			Temperature			Interaction		
	F	d.f.	P	F	d.f.	P	F	d.f.	P
Feeding rate zoea I	29.612	1	**<0.0001**	6.611	3	**0.0012**	1.583	3	0.2114
Feeding rate zoea II	116.32	1	**<0.0001**	55.39	2	**<0.0001**	16.21	2	**<0.0001**
Survival of starved zoea I	0.6109	1	0.4396	21.54	3	**<0.0001**	2.227	3	0.1018
Survival of starved zoea II	3.423	1	0.0723	8.896	3	**0.0001**	0.568	3	0.6393

Figures

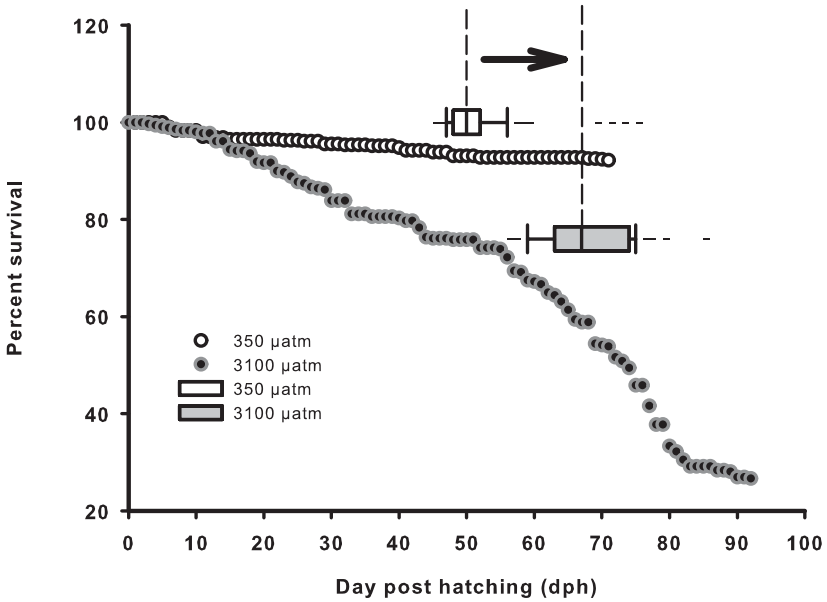


Fig. 1 Survival (%) of zoea I larvae of *H. araneus* reared under 350 µatm CO_2 (control conditions, white circle) and 3100 µatm CO_2 (black circle) after the two weeks of the respective pre-exposure of ovigerous females and eggs. Data were collected from hatching onward until larvae were either dead or moulted to the second stage. Box whisker plots show developmental time of zoea I larvae of *H. araneus* reared under 350 µatm CO_2 (white) and 3100 µatm CO_2 (grey) from hatching until moulting to the second stage. Box limits represent 25th and 75th percentiles, the line within the box marks the median and whiskers indicate 90th and 10th percentiles. Outlying data points are marked by black dots. The arrow indicates the shift of developmental time of larvae reared at high seawater PCO_2 in comparison to control larvae.

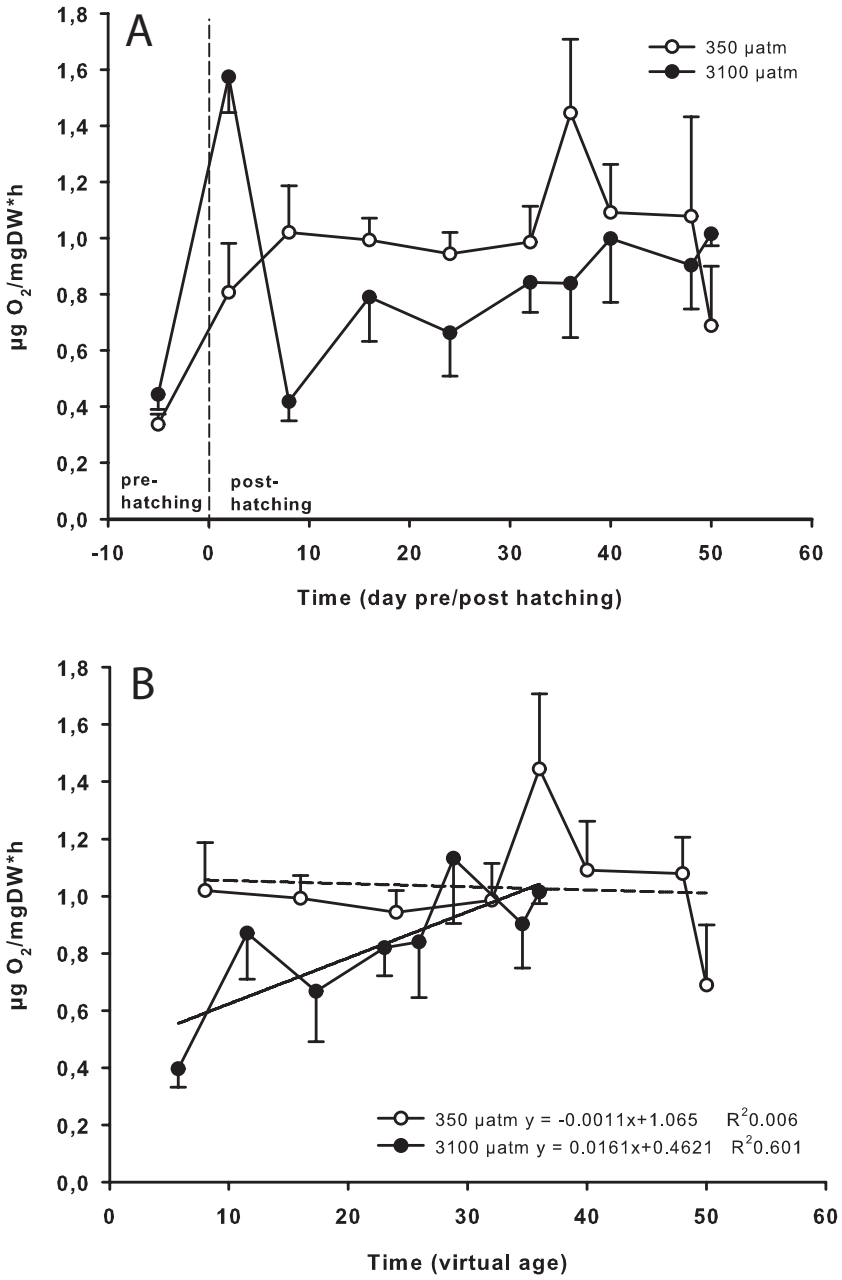


Fig. 2 A Weight specific oxygen consumption of embryos (pre-hatching) and zoea I larvae (post-hatching) of *H. araneus* reared under two different levels of seawater P_{CO_2} (350 µatm CO_2: white circles; 3100 µatm CO_2: black circles). Mean±SE. B Relationship between virtual age (days) and weight specific oxygen consumption (µg O_2/mgDW*h) of *H. araneus* zoea I larvae reared under two different seawater P_{CO_2} (350 µatm CO_2: white circles and dashed regression; 3100 µatm CO_2: black circles and solid regression). N=8

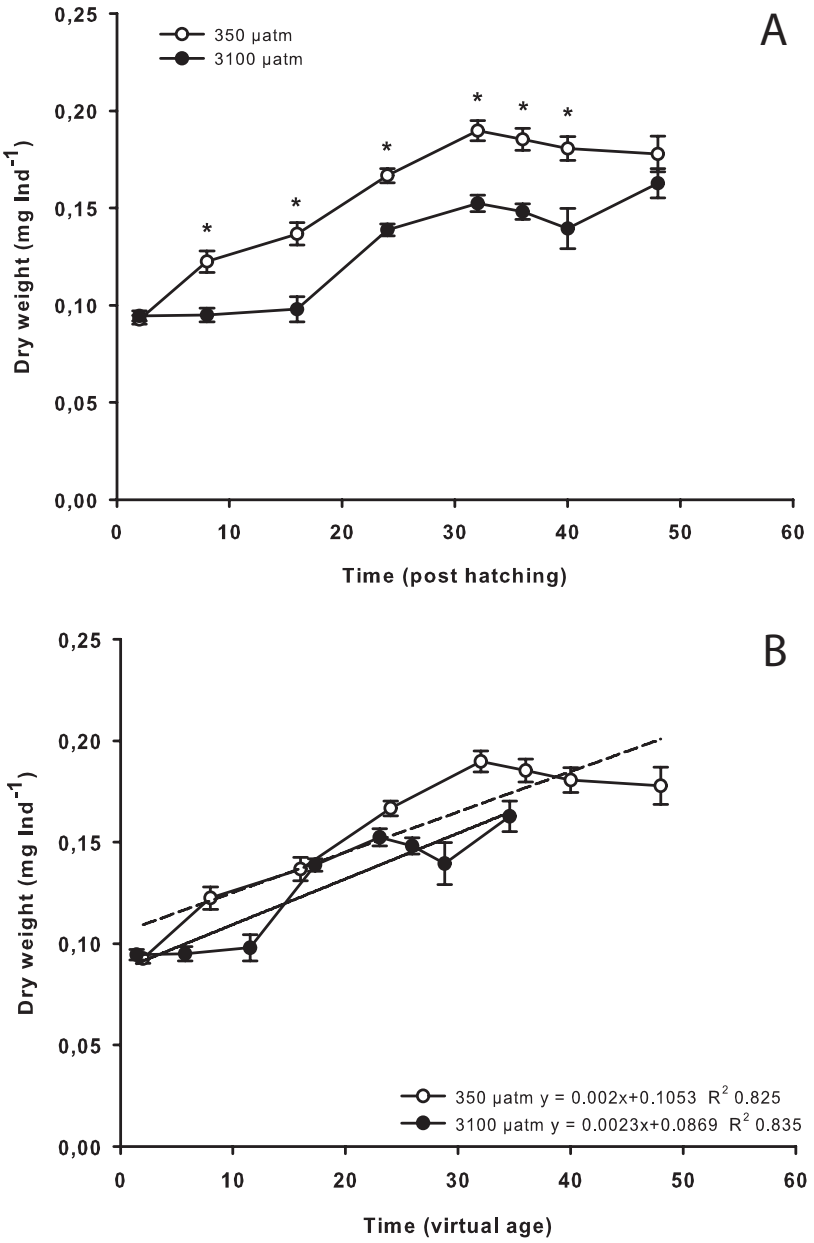


Fig. 3 A Dry weight (mg Ind $^{-1}$) of zoea I larvae of *H. araneus* reared under two different seawater P_{CO_2} levels (350 µatm CO_2: white circles; 3100 µatm CO_2: black circles) during time of development. Mean±SE. Asterisks indicate significant differences between treatments on the same developmental day. B Relationship between virtual age (days) and dry weight (mg Ind^{-1}) of *H. araneus* zoea I larvae reared under two different seawater P_{CO_2} (350 µatm CO_2: white circles and dashed regression; 3100 µatm CO_2: black circles and solid regression). N=8

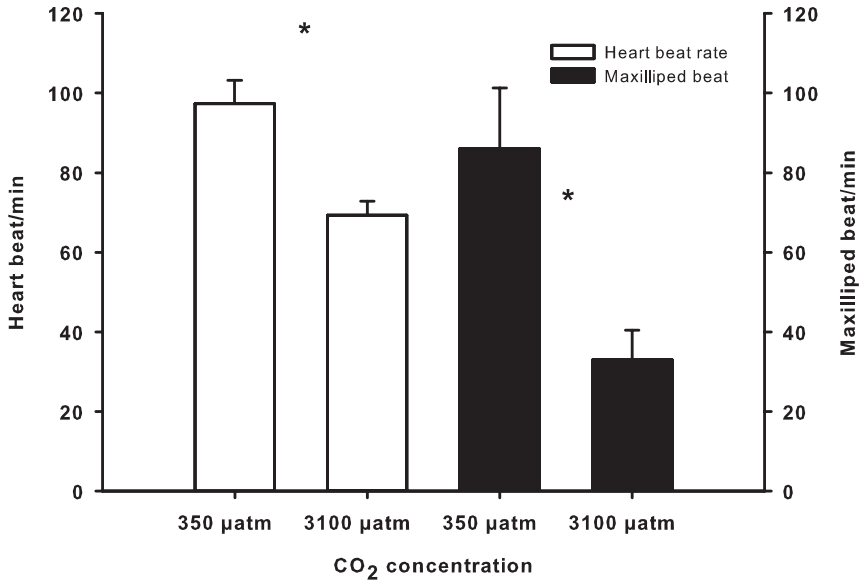


Fig. 4 Heart beat rate (white bar) and maxilliped beat rate (black bar) of 50 day old zoea I larvae of *H. araneus* reared under two different seawater P_{CO_2} (350 µatm CO_2, 3100 µatm CO_2). Mean±SE. Asterisks indicate significant differences between treatments. N=5

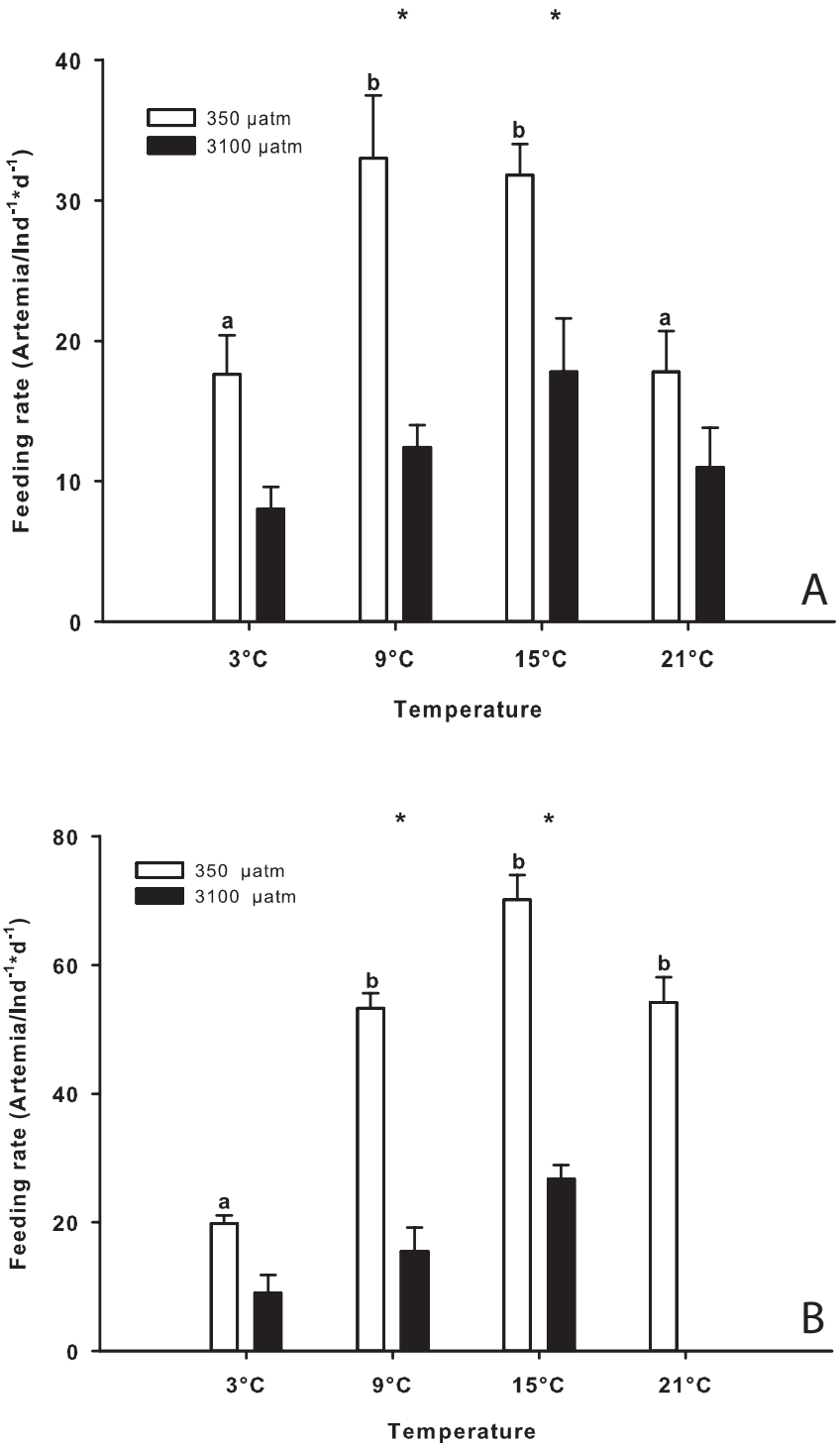


Fig. 5 Feeding rate (Artemia/Ind^{-1}*d^{-1}) of zoea I larvae (A) and zoea II (B) of *H. araneus* reared under two different seawater PCO_2 (350 µatm CO_2: white bars; 3100 µatm CO_2: black bars) at different temperatures. Mean±SE. Asterisks indicate significant differences between treatments at the same experimental temperature. Different letters indicate significant differences between temperatures within one treatment. N=6

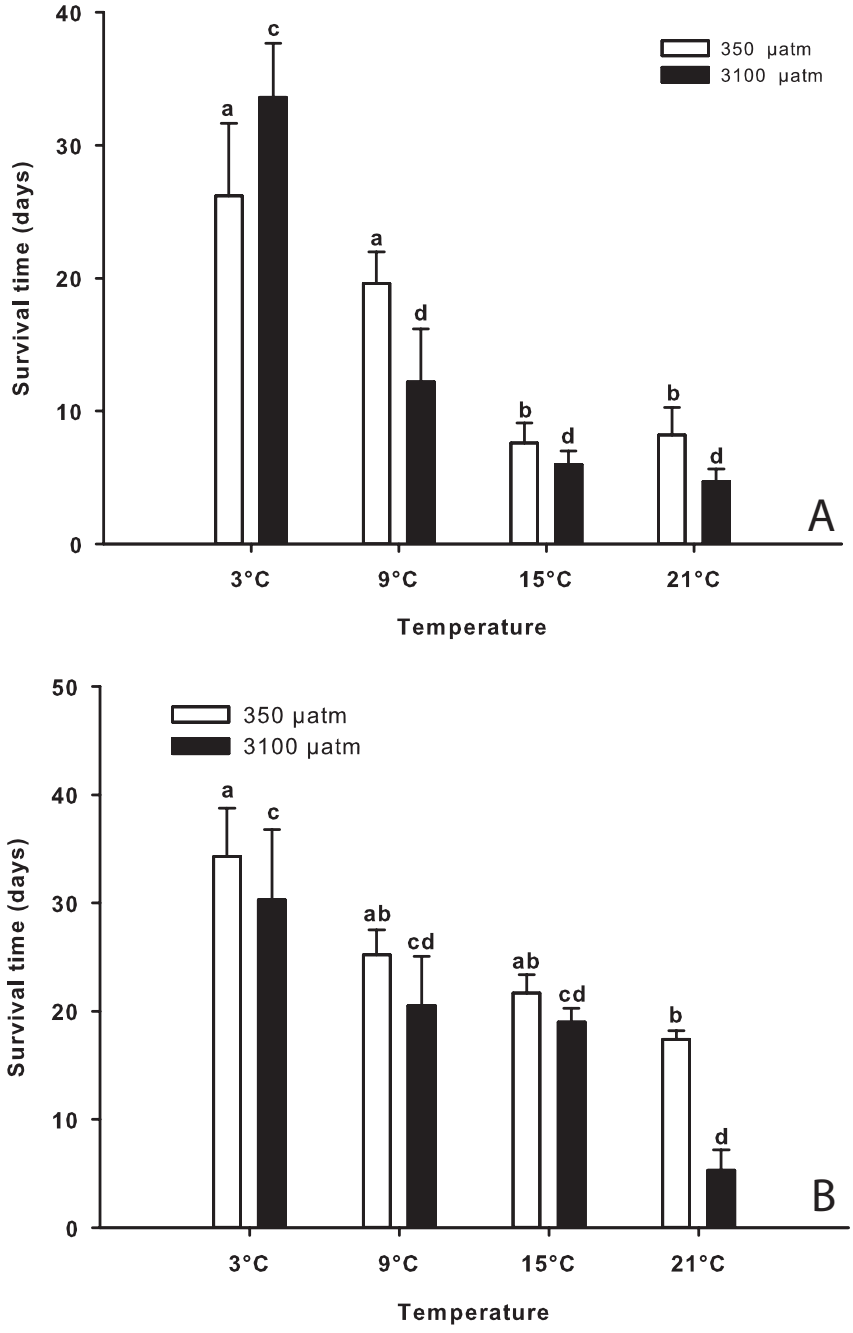


Fig. 6 Survival time (days) of starved zoea I larvae (A) and zoea II (B) of *H. araneus* reared under two different seawater P_{CO_2} (350 µatm CO_2: white bars; 3100 µatm CO_2: black bars) at different temperatures. Mean±SE. Different letters indicate significant differences between temperatures within one treatment. N=6

Publication III

Temperature tolerance of different larval stages of the spider crab *Hyas araneus* exposed to elevated seawater PCO_2

Schiffer M, Harms L, Lucassen M, Mark FC,
Pörtner HO and Storch D

Temperature tolerance of different larval stages of the spider crab *Hyas araneus* exposed to elevated seawater P_{CO_2}

Melanie Schiffer*, Lars Harms, Magnus Lucassen, Felix C. Mark, Hans O. Pörtner and Daniela Storch

Integrative Ecophysiology, Alfred-Wegener-Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

* Email: Melanie.Schiffer@awi.de, Phone: +49 (471) 4831-1380

Abstract

Exposure to elevated seawater P_{CO_2} limits the thermal tolerance of adult crustaceans. However, the underlying mechanisms have not been comprehensively explored. Larval stages of crustaceans are even more sensitive to environmental hypercapnia and possess narrower thermal windows than adults. In a mechanistic approach, we analysed the impact of high seawater CO_2 on parameters at different levels of functional hierarchy, from the molecular to the whole animal level. At the whole animal level we measured oxygen consumption, heart rate and activity during acute warming in zoea and megalopa larvae of the spider crab *Hyas araneus* exposed to different levels of seawater P_{CO_2}. Furthermore, the expression of genes responsible for cellular stress/heat shock response, acid-base regulation and mitochondrial energy metabolism was analysed before and after larvae were heat shocked. Zoea larvae showed a high heat tolerance, which decreased at elevated seawater P_{CO_2}, while the already low heat tolerance of megalopa larvae was not limited further by hypercapnic exposure. There was a combined effect of elevated seawater CO_2 and heat shock in zoea larvae causing elevated transcript levels of heat shock proteins. This reflects the downward shift in thermal limits and indicates a capacity to improve passive thermal tolerance under CO_2. In all three larval stages, hypercapnic exposure elicited an up-regulation of genes involved in oxidative phosphorylation, which was not accompanied by increased energetic demands. An up-regulation of these enzymes might be due to limited enzyme activities through bicarbonate inhibition and could be important to maintain standard metabolic rates at high seawater CO_2 levels. The present study underlines the necessity to align transcriptomic data with physiological responses to address underlying mechanisms affected by an interaction of elevated seawater P_{CO_2} and temperature extremes.

Introduction

The world's ocean covers about 71 percent of the earth surface and supports nearly 50 percent of all species on earth. Its surface waters directly interact with the atmosphere and are affected by atmospheric fluctuations. Thus, anthropogenic increases of the temperature and CO_2 content of the atmosphere will also affect the oceans. Atmospheric temperatures are predicted to rise 0.2°C per decade during the next two decades (IPCC 2007), and the concentration of atmospheric CO_2 may reach levels of 2000 ppm by 2300, leading to a drop in surface water pH by up to 0.8 pH units (Caldeira & Wickett 2005). Marine organisms will thus have to cope with concomitant changes in seawater temperature and pH. Combined or interactive effects of these environmental factors on the physiology of marine organisms can result from the same physiological mechanisms being affected by both factors (Pörtner et al. 2005).

To address the question of how organisms deal with thermal challenges, the concept of oxygen and capacity limited thermal tolerance (OCLTT) has been developed (Pörtner 2001, Pörtner and Farrell 2008). The concept is based on the assumption that a mismatch between oxygen demand and oxygen supply due to limited capacity of ventilatory and circulatory systems at temperature extremes limits the aerobic scope and, as a consequence, thermal tolerance (Frederich and Pörtner 2000). The earliest, ecologically relevant, thermal tolerance limits are set by the pejus temperatures (T_p), while beyond pejus range a critical temperature (T_c) defines individual physiological tolerance. In the pejus temperature range, heartbeat and ventilation are increasing with temperature to compensate for the rise in oxygen demand in the warmth. (Frederich and Pörtner 2000, Pörtner 2001, Wittmann et al. 2008, Storch et al. 2011). Beyond the T_p, haemolymph oxygen partial pressure decreases as a result of limited capacities of ventilation and circulation entailing a progressive mismatch between oxygen demand for maintenance and oxygen supply (Walther et al. 2009). In warm temperate species, hypoxia occurs and, finally, anaerobic metabolism sets in at the critical temperature. Survival beyond T_c is time-limited (Pörtner and Zielinski 1998, Frederich and Pörtner 2000). At the upper end of the thermal tolerance window, denaturation temperature might elicit a loss of protein function, the heat shock response and oxidative stress (Pörtner 2001).

The mechanistic interactions of elevated seawater P_{CO_2} and temperature extremes have been proposed to narrow the thermal tolerance window of an organism exposed to high CO_2 levels (Pörtner and Farrell 2008; Pörtner 2008). Dependent on elevated seawater CO_2

concentration, upper thermal tolerance limits have been observed to be lowered by several °C in adult crustaceans (Metzger et al. 2007; Walther et al. 2009). Zittier et al. (2012) found elevated seawater P_{CO_2} and heat stress to act synergistically reducing the righting response in the spider crab *Hyas araneus*. Elevated seawater P_{CO_2} also limits cold-tolerance of marine invertebrates: in cold-exposed king scallops, *Pecten maximus*, mortality rates increased from 55 % under normocapnia to 90 % under hypercapnia (Schalkhausser et al. 2012).

To understand the synergistic effects of increasing seawater P_{CO_2} and temperature on a population level, it is important to include the most vulnerable life cycle stages. Early developmental stages are suggested to be most sensitive to environmental hypercapnia (Kurihara 2008) and to possess narrow thermal windows (Pörtner and Farrell 2008; Pörtner 2010). They might, thus, be a bottleneck for successful survival and viability of a species in a warm and high CO_2 ocean. So far, there is limited data available on the thermal tolerance of larval stages exposed to elevated seawater P_{CO_2}.

Sensitivities of marine organisms to ocean acidification are linked to efficient acid-base regulation of intra- and extracellular compartments. Whereas intracellular pH is typically restored rapidly, extracellular pH disturbances are often compensated only partially (Pörtner et al. 1998). Capacities for acid-base regulation are related to taxon, environmental background and adaption of a species. While the shallow-living crab *Cancer magister* is able to fully compensate hypercapnia induced haemolymph acid-base disturbances, haemolymph acidosis appears almost uncompensated in the deep-sea crab *Chionoecetes tanneri* (Pane and Barry 2007). Commonly, fish have higher capacities to compensate for acid-base disturbances than invertebrates (Pörtner et al. 2004). It is known for fish that environmental temperature can also affect the acid-base response. Rainbow trout acclimated to high environmental temperature showed a more severe blood acidosis after exercise than fish kept at a lower temperature (Kieffer et al. 1994). Due to the interactive effect of CO_2 and temperature on the acid-base status, strong acid-base disturbances that lead to reduced protein function may be responsible for a lower temperature tolerance in organisms exposed to elevated CO_2. An uncompensated drop in extracellular pH (pH_e) due to elevated seawater P_{CO_2} can have a depressing effect on aerobic energy metabolism of some tissues like muscle (Reipschläger and Pörtner 1996) and isolated liver cells (Langenbuch and Pörtner 2003) indicated by reduced oxygen consumption and will likely reduce the capacities to withstand temperature extremes. In contrast, a study by Chapman et al. (2011) indicates an increase in the capacity for ATP

generation under low pH and elevated temperature by elevating transcripts involved in mitochondrial oxidative phosphorylation in the eastern oyster *Crassostrea virginica*, while transcripts involved in protein synthesis and cell growth were reduced.

One of the most important groups of proteins involved in the prevention of protein denaturation under stressful environmental conditions are heat shock proteins (HSP). These chaperones are relevant for normal cellular homeostasis as well as for preventing cellular damage during cellular stress, e.g. temperature stress (HSP70 and HSP90 families) (Kregel 2002). Although HSP70 expression is generally more associated with temperature than pH stress, HSP70 was found to be up-regulated at low environmental pH in the eastern oyster *Crassostrea virginica* (Chapman et. al 2011). In contrast, HSPs were down-regulated in sea urchin larvae and larvae showed a reduced and delayed HSP70 response at elevated seawater P_{CO_2} (O'Donnell et al. 2009; Todgham and Hofmann 2009). Increased HSP production as a consequence of the synergistic effects of high CO_2 and high temperature could be an energy consuming process, while a reduced HSP response would lead to increased protein damage.

Studies addressing temperature tolerance of marine organisms exposed to high seawater P_{CO_2} focused either on whole animal performance or transcriptomic response (Walther et al. 2009; Zittier et al. 2012; Metzger et al. 2007; Chapman et al. 2011). Studies aligning transcriptomic data to physiological responses are scarce (Stumpp et al. 2011a,b). However, integrating gene expression analysis in a physiological study will improve our understanding of how mechanistic interactions of high temperature and elevated seawater P_{CO_2} influence animal performance.

The aim of the paper is to investigate the effect of elevated seawater P_{CO_2} on the heat tolerance of the three larval stages of the spider crab *Hyas araneus*. *Hyas araneus* is a shelf species and has a wide distribution range from temperate to Arctic waters (Christiansen 1969). Larvae go through two zoea stages and one megalopa stage before settling into the adult habitat. In a mechanistic approach, we analysed parameters on different levels of functional hierarchy, from the molecular to the whole animal level. As temperature tolerance of adult *Hyas araneus* has been shown to be reduced by high CO_2 (Walther et al. 2009) and larvae are supposed to be more sensitive to synergistic effects of CO_2 and temperature (Pörtner and Farrell 2008), larvae were exposed to high seawater CO_2 of 3300 µatm and temperature extremes (10°C above rearing temperature) to study mechanisms affected by both factors and the interaction between these factors. At the

whole organism level, we measured active metabolic rate, heart rate and larval activity during continuous warming in the three larval stages reared at different seawater P_{CO_2} to identify differences in heat tolerance between CO_2 treatments and stages.

CO_2 and temperature induced shifts in gene expression were studied in batches of larvae of each stage by sampling directly from the different CO_2 treatments and after exposure to short term heat shock. Expression of genes responsible for cellular stress/heat shock response as a protection process, acid-base regulation as an important energy consuming process (Pörtner et al. 2000a) and mitochondrial energy metabolism as an energy supplying process, was analysed. These processes are hypothesized to be of central importance for a limitation in thermal tolerance during hypercapnic exposure. Exposure to elevated seawater P_{CO_2} affects the gene expression of heat shock proteins of larvae and adult marine ectotherms with varying responses from reduced capacity of cellular stress responses (Todgham and Hofmann 2009; O'Donnell et al. 2009), to up-regulation of heat shock proteins at low pH (Chapman et al. 2011).

The capacities to regulate hypercapnia-induced blood acid-base disturbances by means of ion transporters might prevent strong acid-base disturbances that could lead to reduced protein function and lower temperature tolerance. Systemic hypercapnia also causes metabolic depression by lowering pH (Reipschäger and Pörtner 1996) accompanied by increasing gas partial pressure gradients (Pörtner et al. 1998) and will reduce the organisms' capacity to increase its rate of aerobic energy turnover (Pörtner et al. 2005). A metabolic depression under hypoxia was mirrored in the repression of genes in the mitochondrial citric acid cycle and in the electron transport system in gills of adult zebrafish (van der Meer et al. 2005).

With our data, we have been able to align molecular responses to whole organism performance and to reveal mechanisms affected by the putative synergistic action of high CO_2 and high temperature.

Material and methods

Larval collection and maintenance

Ovigerous females of *Hyas araneus* were collected by local fishermen in Gullmarsfjorden (west coast of Sweden, 32 PSU, 15°C) in September 2010 and transferred to the Alfred Wegener Institute in Bremerhaven. They were maintained in flow-through aquaria at 10°C,

32 PSU and a constant dark: light cycle (12h: 12h). During larval hatching, which started in June 2011, twelve females were placed individually in 2 l aquaria to collect larvae of each female separately. Equal numbers of newly hatched larvae of the twelve females were pooled and subsequently transferred into 0,5 l enclosed culture vessels at a density of 30 individuals per vessel for the zoea larvae. The density was reduced to 15 larvae for the bigger megalopa stage. All experiments were conducted with larvae that had hatched within 24 h. They were reared in enclosed culture vessels filled with seawater of different CO_2 concentrations at a constant temperature of 10.0 ± 0.5°C and a salinity of 31.8 PSU (450 µatm: control treatment; 3300 µatm: high CO_2 treatment). Zoea I that moulted into the zoea II stage or zoea II that moulted into the megalopa stage at the same day were pooled together into another culture vessel at a maximum of 30 zoea larvae or 15 megalopa larvae, respectively. Seawater was provided from reservoir tanks (60l) at 10.0 ± 0.5°C and a salinity of 31.8 PSU, continuously bubbled with an air/CO_2 mixture using a mass flow controller (HTK Hamburg GmbH, Germany). Seawater in culture vessels and food (freshly hatched *Artemia* sp. nauplii, Sanders Brine Shrimp Company, Ogden, Utah, USA) were changed daily and dead larvae and moults were removed. Water physicochemistry was monitored by measuring temperature, salinity and pH (NBS scale, pH_{NBS}, corrected by Dixon buffered seawater) and the collections of water samples for the determination of dissolved inorganic carbon (DIC). Water P_{CO_2} was calculated from DIC, pH_{NBS}, temperature and salinity using the program CO_2SYS (Lewis and Wallace, 1998) (table 1).

Determination of the larval thermal tolerance window

All experiments were conducted during the middle of larval development with 9-13-day old zoea I and zoea II larvae and 14-18-day old megalopa larvae as thermal tolerance might change with development time. Measurements started at the rearing temperature of 10°C. After each measurement temperature was increased to the next experimental temperature by 3°C in 30min. Experimental temperatures were 10°C, 13°C, 16°C, 19°C, 22°C, 25°C and 28°C. At each temperature, oxygen consumption, heart rate and maxilliped beat rate were measured in the various larval stages.

Oxygen consumption

Oxygen consumption rates of individual larvae were measured in closed, double-walled respiration chambers (OXY041 A, Collotec Meßtechnik GmbH, Niddatal, Germany). Chambers were connected via tubing to a thermostatted water bath to control

temperature. Oxygen saturation was recorded by oxygen micro-optodes (NTH-PSt1-L5-TF-NS*46/0,80-YOP, PreSens GmbH, Regensburg, Germany), connected to a Microx TX3 oxygen meter (PreSens GmbH, Regensburg, Germany).

For measurements, the larvae were transferred into the respiration chamber. After each measurement, the next experimental temperature was established within half an hour. Between each measurement during adjustment of the new experimental temperature, larvae were maintained in culture vessels containing seawater of the corresponding CO_2 concentration, which were placed in the thermostatted water bath to increase the temperature according to the experimental protocol. Afterwards larvae were allowed to acclimate for half an hour before being transferred to the respiration chamber. The plunger of the chamber lid was inserted and the volume of the chamber was reduced to 150 µl. The needle of the micro-sensor was inserted into the chamber through a hole in the lid and the sensitive tip of the optode was placed in the middle of the chamber. Respiration measurements were carried out for thirty minutes. Before each measurement, blanks were run to consider bacterial oxygen consumption. Larval oxygen consumption was expressed as $\mu gO_2 * mg\ DW^{-1} * h^{-1}$ to allow for treatment-specific differences in larval dry weight. For all larval stages, at least six larvae from each CO_2 treatment were used to measure oxygen consumption. Individual larvae were measured at each experimental temperature.

After respiration measurements at the highest experimental temperature of 28°C, larvae were removed from the chamber and briefly rinsed with deionized water and blotted dry. For dry weight determination, larvae were stored at -20°C in pre-weighed tin cartridges, freeze-dried over night and subsequently weighed on a high precision balance (Mettler Toledo AG, Greifensee, CH-8606, CH).

Heart rate and maxilliped beat rate

Heart rates of individual larvae were measured according to Storch *et al*. (2011). Heart rate was recorded using a digital camera (AxioCam MRm, Carl Zeiss, Mikroimaging GmbH, Göttingen, Germany) mounted onto a microscope (Axio Observer A1, Carl Zeiss). Larvae were placed under the scope in a temperature-controlled flow-through micro-chamber (built at Alfred Wegener Institute, Bremerhaven, Germany) filled with seawater of the corresponding CO_2 concentration, which allowed changing the temperature according to the experimental protocol without disturbing the larvae. Temperature controlled seawater (10°C, 32PSU) was provided from a reservoir vessel placed in the thermostatted

water bath and was pumped through the chamber with a flow rate of 5ml/min to avoid a decrease in oxygen concentration due to larval respiration. Before closing the chamber, larvae were positioned in the centre of the micro-chamber by gluing the carapace to a thin glass spine, which itself was attached to a glass table. Larvae were left for 1 h to recover from handling stress and were videotaped for 1 min. Afterwards temperature was changed according to the protocol described above and at each experimental temperature the larvae were videotaped for 1 min. The video sequence was analysed for heart and maxilliped beat rates, respectively, by counting the beats min^{-1}. The beating heart can easily be seen through the transparent carapace. Heart rate and maxilliped beat rate was calculated for each larva as the mean number of beats min^{-1} ± SE from three 10s intervals. For all larval stages, five larvae from each CO_2 treatment were used to measure heart rates. The same five individual larvae were used to calculate maxilliped beat rates. Individual larvae were measured at each experimental temperature. Unfortunately, no data on pleopod beat rate of the megalopa stage could be obtained, as pleopod beating was too inconsistent for calculations.

Gene expression patterns

Sampling

Samples were taken on day 0 and day 15 post hatching in zoea I larvae, on day 3 and day 15 post moulting in zoea II and on day 3 in megalopa larvae. These time points were chosen to analyse hypercapnia-induced changes in gene expression at different time point within the larval development as CO_2 sensitivities might change with development time. On day 15, gene expression can be aligned to whole organism performance. Unfortunately, no data on gene expression could be obtained for the megalopa stage on day 15 due to loss of samples during RNA isolation. At each time point batches of 15 to 20 larvae (depending on larval stage) were transferred into 1.5 ml Eppendorf tubes containing RNAlater (Ambion, Austin, TX) and stored at -80°C. One batch of larvae from each CO_2 treatment was sampled directly from the culture vessel, while a second batch from each CO_2 treatment was heat shocked by transferring the larvae from the rearing temperature of 10°C into a 2l glass jar containing seawater of 20°C and the corresponding CO_2 concentration. The glass jar was placed in a thermostatted water bath to keep the temperature constant. After 5 hours at 20°C larvae were sampled and frozen as described above. Each treatment (control/high CO_2 concentration at 10°C and 20°C, respectively) was replicated five times and for each replicate isolation of RNA and real-time PCR was conducted.

Isolation of RNA

Frozen samples were thawed and larvae were transferred from RNAlater into homogenisation buffer (Qiagen, Hilden, Germany). Larvae were homogenized in a Precellys homogenizer (Bertin Technoligies, France) using 2ml homogenisation tubes. Afterwards total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany) following the manual. Extracted RNA was solubilized in 0.1 mM EDTA and 10 mM Tris and RNA purity and concentration were determined using a Thermal Scientific Nanodrop 2000 spectrometer.

Quantitative real-time PCR

10 µg of total RNA was treated with DNAse (Turbo DNA-free, Ambion) in order to digest genomic DNA remnants and RNA concentration was measured again (NanoDrop). Subsequently, 0.4 µg total RNA was subject to cDNA synthesis using the High capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Darmstadt, Germany). Expression of 20 important genes involved in mitochondrial energy metabolism, acid-base regulation and stress/heat shock response, were analysed because they were assumed to be affected by synergistic effects of temperature and CO_2. Furthermore, tubulin was chosen as potential housekeeping candidate. Primers (table 2) for 21 genes were designed using the Primer Express software for real-time PCR (version 3.0, Applied Biosystems, Darmstadt, Germany). Sequences were obtained from the recently in our lab established transcriptome of Sub-Arctic *Hyas araneus* (Harms et al. submitted). The PCR was performed using a 7300 Real Time PCR System (Applied Biosystems) and the Sybr green qPCR master mix (Fermentas). For all primers, PCR efficiency was examined by using six different dilutions of template cDNA (1:20; 1:40; 1: 100; 1:200; 1:1000; 1:2000). Efficiency was estimated by 10^(-1/slope) and was for all primers > 1.9 (table 2). Primer concentrations were 300 nM and all genes were amplified with 1 ng of template cDNA. After amplification, a melting curve was acquired to verify the specific amplification of fragments.

C_t values of all genes were transformed into quantities (Q) using the formula $Q = E^{(-Ct)}$ with E being the reaction specific efficiency and C_t being the C_t value determined by the 7300 Real Time PCR System. The analysis by geNorm plus suggested VATPase (VA) and sodium hydrogen exchanger (NHE) as the most stable expressed genes in all treatments (VA M= 0.310; NHE M=0.325). A normalization factor was determined by calculating the geometric mean of these two reference genes. Quantities of each sample for each gene were divided

by the appropriate normalisation factor.

Statistical analysis

Results were analysed using SigmaPlot (Version 12, Systat Software, Inc., San Jose, California). All data were checked for outliers by Nalimov's test (Noack 1980). A two-way ANOVA was used to investigate effects of CO_2 concentration and temperature on larval oxygen consumption, heart rate and maxilliped rate. Tukey's multiple comparison tests were used for *a posteriori* analyses. When a disordinal interaction between factors was detected, one-way ANOVAs were run additionally for each CO_2 concentration to detect differences among temperatures. Holm Sidak tests were used for *a posteriori* analyses. When data did not meet assumptions for one-way ANOVA, a Kruskal-Wallis one-way ANOVA of Ranks was conducted and Dunn's tests were used for *a posteriori* analyses.

An unpaired t-test was conducted to analyse the effect of temperature on gene expression in zoea I on day 0. When data did not meet assumptions for an unpaired t-test, a Mann-Whitney Rank Sum test was run. Two-way ANOVAs were applied to analyse the effect of CO_2 concentration and temperature on gene expression for each time point within the different larval stages. Tukey's multiple comparison tests were used for *a posteriori* analyses. When a disordinal interaction between factors was detected, one-way ANOVAs including all different treatments were run additionally followed by a Holm Sidak test for multiple comparisons. When data did not meet assumptions for one-way ANOVA, Kruskal-Wallis one-way ANOVA of Ranks was conducted followed by Dunn's test for multiple comparisons.

A canonical correspondence analysis was applied (Brodgar, Highland Statistics Ltd., Newburgh, UK), to ascertain whether the different factors temperature, seawater PCO_2 and life stage influence gene expression.

Results

Determination of the larval thermal tolerance window

All significant interaction between temperature and CO_2 concentration detected by the two-way ANOVA (table 3) can be described as disordinal, which renders an interpretation of both main factors (CO_2 and temperature) impossible.

Oxygen consumption

Oxygen consumption of zoea I larvae increased with temperature, but not with seawater CO_2 concentration (2 way-ANOVA, table 3). *A posteriori* tests identified peaks in oxygen consumption at 25°C in control larvae (2.3 ± 0.3 μO_2 mg DW^{-1}* h^{-1}) and at 22°C in high CO_2 larvae (2.2 ± 0.4 μO_2 mg DW^{-1}* h^{-1} (Fig 1). At 28°C larval oxygen consumption showed a significant decrease even below values observed at 10°C for both CO_2 treatments (Fig. 1). Oxygen consumption was significantly lower under high seawater PCO_2 at 25°C in comparison to oxygen consumption of control larvae.

There was a significant interaction between temperature and CO_2 concentration (table 3). The interaction was significant because larvae kept at high CO_2 showed lower respiration rates at 25°C in comparison to larvae from the control treatment (Fig. 1B). One-way ANOVAs run for each CO_2 concentration individually showed a significant effect of temperature on zoea II respiration rates for both CO_2 treatments. Oxygen consumption of control larvae increased between 10°C and 19°C, remained constant between 19°C and 25°C followed by a significant decrease at 28°C (Fig. 1B). In contrast, oxygen consumption of larvae reared at elevated CO_2 remained constant between 13°C and 22°C and showed a sharp decrease already at 25°C.

There was a significant interaction in megalopa larvae (table 3). Individual one-way ANOVAs showed a significant effect of temperature on oxygen consumption rates of megalopa larvae reared at control PCO_2, while there was no effect of temperature on oxygen consumption of high CO_2 larvae (table 3). *A posteriori* tests determined constant respiration rates between 10°C and 22°C and a significant decrease at 28°C for megalopa kept at control conditions (Fig. 1C). Oxygen consumption was significantly lower under high seawater PCO_2 at 22°C in comparison to oxygen consumption of control larvae. The highest oxygen consumption was found at 22°C in control larvae (1.9 ± 0.5 μO_2 mg DW^{-1}* h^{-1}) and at 13°C under high seawater PCO_2 (1.6 ± 0.7 μO_2 mg DW^{-1}* h^{-1}).

Heart rate

The heart rate of zoea I larvae was significantly affected by temperature, but not by seawater CO_2 concentration (two-way-ANOVA, table 3). For zoea I reared under control conditions heart rate increased between 10°C and 25°C with highest heart rates at 25°C (418 ± 14 beats min^{-1}, Fig. 2A). A similar increase between 10°C and 25°C could be seen under high seawater PCO_2 with highest heart rates of 353 ± 54 beats min^{-1} at 25°C. Upon further warming to 28°C there was a significant decrease of heart rate in both treatments

to 133 ± 70 beats min^{-1} at control conditions and 153 ± 46 beats min^{-1} under high seawater P_{CO_2}.

There was a significant interaction between temperature and CO_2 concentration in zoea II larvae (table 3). The interaction was significant because larvae showed lower heart rates at 25°C in the high CO_2 treatment (Fig. 2B). One-way ANOVAs were run individually for both CO_2 treatments and showed a significant effect of temperature on zoea II heart rates (table 3). Holm-Sidak test identified a significant increase of zoea II heart rates between 10°C and 25°C in control larvae and between 10°C and 22°C in high CO_2 larvae, respectively. Subsequently, heart rates decreased at 28°C in zoea II kept at control seawater P_{CO_2}. In contrast, a significant decrease of heart rates could already be observed at 25°C followed by a further decrease at 28°C in larvae reared at elevated P_{CO_2} (Fig. 2B).

The heart rate of megalopa larvae was significantly affected by temperature, but not by seawater CO_2 concentration (two-way-ANOVA, table 3). Heart rates remained constant between 10°C and 22°C followed by a significant decrease between 19°C or 22°C and 25°C in larvae from high CO_2 and control treatment, respectively (Fig. 2C). At 28°C no heart beat could be detected at either treatment groups.

Maxilliped beat rate

The maxilliped beat rate of zoea I larvae was significantly affected by temperature, but not by seawater CO_2 concentration (two-way-ANOVA, table 3). *A posteriori* Tukey tests revealed constant maxilliped beat rates between 10°C and 25°C and a decrease upon further warming to 28°C, which was significant between 16°C and 28°C in zoea I larvae reared at control conditions. There was no significant difference between maxilliped beat rates at different temperatures in high CO_2 zoea I larvae (Fig. 3A).

A significant effect of temperature, but not of seawater CO_2 concentration on maxilliped beat rates of zoea II larvae was detected by a two-way ANOVA (table 3). Zoea II maxilliped beat rates decreased with increasing temperature in larvae from both seawater CO_2 concentrations (*a posteriori* Tukey tests) (Fig.3B). Zoea II maxilliped beat rates stayed the same between 10°C and 22°C under control conditions in larvae from both seawater CO_2 concentrations. Furthermore, there was no significant difference between 22°C and 25°C in control larvae, while there was a significant drop in maxilliped beat rates in larvae reared at elevated seawater P_{CO_2}. Zoea II stopped maxilliped beating at 28°C under control conditions while no beating was detected at 25°C and 28°C under high seawater P_{CO_2}. A

significant difference between maxilliped beat rates of zoea II larvae reared at different seawater CO_2 levels was indicated at 19°C (*a posteriori* Tukey tests) with higher maxilliped beat rates in larvae reared under elevated seawater P_{CO_2}.

Gene expression patterns

For the purpose of clarity only significant changes in gene expression will be reported and discussed (table 5). Significant effects of seawater CO_2 concentration, heat shock and a combination of both factors on expression of genes responsible for cellular stress/heat shock response, acid-base regulation and mitochondrial energy metabolism will be reported separately. We presumed a combined effect when both factors, heat shock and seawater CO_2, significantly affected larval gene expression (table 5).

Cellular stress/heat shock response

Seven different sequences, identified as heat shock proteins (HSP) by Blastx (E-Value cut-off of $1E^{-3}$), were selected to investigate effects of elevated seawater CO_2 and heat shock on HSP gene expression in the different larval stages, among those 4 representatives of the HSP 70 family (HSP70_1-4), HSP 90, HSP 26 and HSP 60.

Heat shock effect

There was a significant interaction between heat shock and seawater CO_2 concentration for gene expression of HSP70_1, HSP70_4 and HSP90 in zoea II larvae on day 3 and day 15 and for HSP70_4 and HSP90 in megalopa larvae.

A heat shock of 20°C significantly increased the expression of HSP70_1 in zoea I on day 0 ($p = 0.020$), just as it was found on day 15 (ANOVA II: $F_{1,16} = 162.293$, $p < 0.001$). In zoea II larvae on day 3 and day 15, mean values among the treatment groups were significantly different (table 4) with higher HSP70_1 expression after the heat shock of 20°C. Expression of HSP70_1 was also significantly higher in the megalopa stage after the heat shock (ANOVA II: $F_{1,16} = 42.833$, $p < 0.001$).

Expression of HSP70_2 was influenced significantly by heat shock in the first zoea stage on day 0 (unpaired t test: $p = 0.016$) and day 15 (ANOVA II: $F_{1,15} = 114.379$, $p < 0.001$) with higher expression after the heat shock of 20°C in larvae from both CO_2 treatments. An up-regulation of HSP70_2 expression after the heat shock was also found in the second zoea stage on day 15 (ANOVA II: heat shock, $F_{1,13} = 106.599$, $p < 0.001$) and in the megalopa stage (ANOVA II: $F_{1,16} = 136{,}855$, $p < 0.001$).

HSP70_3 was significantly up-regulated after the heat shock of 20°C in zoea I on day 0 (unpaired t-test: p = 0.010) and on day 15 (ANOVA II: $F_{1,16}$ = 15.610, p = 0.001) and in the second zoea stage on day 3 and day 15 (ANOVA II: day 3 $F_{1,15}$ = 11.462, p = 0.004; day 15 $F_{1,15}$ = 11.462, p = 0.004) as well as in the megalopa (ANOVA II: $F_{1,16}$ = 23.212, p < 0.001).

Exposure to a heat shock increased the gene expression of HSP70_4 of zoea I on day 15 (ANOVA II: $F_{1,16}$ = 29.503, p < 0.001). One-way ANOVAs revealed significant differences in mean values among the treatment groups in zoea II larvae on day 3 (table 4) with increased expression after the heat shock. Heat shock had a significant effect on gene expression in zoea II on day 15 (ANOVA II: heat shock, $F_{1,13}$ = 51.591, p < 0.001) with higher expression levels at 20°C as it could be found in megalopa larvae reared under control conditions.

A significant effect of heat shock on gene expression of heat shock protein 90 (HSP90) could be found in zoea I on day 15 (ANOVA II: $F_{1,16}$ = 33.234, p < 0.001) and in zoea II on day 3 (ANOVA II: $F_{1,14}$ = 7.707, p = 0.015) with higher expression at 20°C in larvae reared at elevated P_{CO_2}. One-way ANOVAs for zoea II on day 15 and megalopa showed differences in mean values of the treatment groups (table 4). Elevated gene expression of HSP90 was found after the heat shock in zoea II and in control megalopa larvae.

The heat shock of 20°C had a significant effect on gene expression of HSP26 in the zoea II on day 15 (ANOVA II: $F_{1,13}$ = 15.298, p = 0.002). A Tukey test revealed a significantly lower gene expression in larvae at an elevated temperature of 20°C.

CO_2 effect

Gene expression of HSP70_4 and HSP26 was significantly affected by seawater CO_2 concentration in zoea I on day 15 (ANOVA II: HSP70_4: $F_{1,16}$ = 13.484, p = 0.002; HSP26: $F_{1,15}$ = 6.498, p = 0,022) with significantly higher expression at 10°C in high CO_2 larvae compared to control larvae.

Combined effect of heat shock and CO_2

Expression of HSP70_1 and HSP70_4 were significantly higher in zoea I on day 15 and zoea II on day 3 after the heat shock of 20°C in larvae reared at elevated seawater P_{CO_2}.

Post hoc analysis revealed an increased expression of HSP70_1-3 and HSP90 at 20°C which in zoea II larvae on day 15 of their exposure to high CO_2 was even higher than in controls.

Acid-base regulation

Four different sequences, identified as acid-base regulation responsible transporters by Blastx (E-Value cut-off of $1E^{-3}$), were selected to investigate effects of elevated seawater CO_2 and heat shock on the acid-base regulation in the different larval stages, among those carbonic anhydrase (CA), sodium potassium ATPase (NKA), sodium bicarbonate cotransporter (NBC) and sodium potassium chloride cotransporter (NKCC).

Heat shock effect

Expression of CA was significantly affected by heat shock in zoea II larvae on day 3 (ANOVA II: $F_{1,16} = 12.967$, $p = 0.002$). A Tukey test examined lower CA expression in high CO_2 larvae after the heat shock. Due to a significant interaction in zoea II on day 15, a one-way ANOVA was conducted and revealed differences between mean values of treatment groups (table 4). Significant down regulation of CA expression after heat shock was found in zoea II larvae reared under control CO_2.

A significant effect of heat shock on gene expression of NKA was found for zoea II on day 3 (ANOVA II: $F_{1,16} = 49.267$, $p < 0.001$) with lower expression after the 5 hrs of heat shock of 20°C.

There was a significant interaction in megalopa for the expression of NKCC. Subsequent one-way ANOVAs exhibited differences in mean values of the treatment groups (table 4) with reduced expression after the heat shock in larvae reared at elevated seawater PCO_2.

CO_2 effect

Expression of CA was significantly affected by seawater CO_2 concentration in zoea II larvae on day 3 (ANOVA II: $F_{1,16} = 6.579$, $p = 0.021$). A Tukey test examined higher CA expression at 10°C in larvae reared at elevated seawater PCO_2. Due to a significant interaction in zoea II on day 15 and the megalopa, one-way ANOVAS were conducted and revealed differences between mean values of treatment groups (table 4). Significant down regulation of CA expression was found in high CO_2 zoea II and megalopa at 10°C in comparison to CA expression of control larvae.

Seawater CO_2 concentration affected NKA expression in zoea II larvae on day 15 (ANOVA: $F_{1,12} = 5.992$, $p = 0.031$) with lower NKA expression in the high CO_2 treatment at 10°C.

Expression of NBC was significantly affected by seawater CO_2 concentration in the zoea I larvae on day 15 (ANOVA II: $F_{1,16} = 12.812$, $p = 0.003$). Expression was significantly reduced after the heat shock of 20°C in larvae reared at elevated PCO_2. Seawater CO_2 concentration affected NBC expression of zoea II on day 3 (ANOVA II: $F_{1,14} = 35.801$, $p < 0.001$) with increased expression in high CO_2 larvae at 10°C and 20°C. Seawater CO_2 concentration affected NBC expression in megalopa (ANOVA II: $F_{1,15} = 6.509$, $p = 0.022$) with higher expression levels in high CO_2 larvae at 10°C.

For megalopa larvae, one-way ANOVAs exhibited differences in mean values of the treatment groups (table 4) with higher NKCC expression at 10°C at elevated seawater PCO_2 in comparison to control larvae.

Mitochondrial energy metabolism

Seven different sequences, identified as enzymes of the mitochondrial energy metabolism by Blastx (E-Value cut-off of $1E^{-3}$), were selected to investigate effects of elevated seawater CO_2 and heat shock on the mitochondrial energy metabolism in the different larval stages, among those pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (IDH), NADH dehydrogenase (NAD), succinate dehydrogenase (SDH), cytochrome c reductase (CCR), cytochrome c oxidase (COX) and ATP synthase (atpA).

Heat shock effect

Expression of PDH was significantly affected by heat shock in zoea II on day 3 (ANOVA II: $F_{1,15} = 9.506$, $p = 0.009$). Larvae reared at control PCO_2 showed significantly lower PDH expression.

Due to a significant interaction in zoea II on day 15 for the expression of NAD, subsequent one-way ANOVAs were conducted showing differences in mean values of treatment groups (table 4) with 7-fold higher NAD expression in control larvae at 20°C.

Expression of SDH was significantly affected by heat shock in zoea II on day 3 (ANOVA II: $F_{1,16} = 14.518$, $p = 0.002$) with lower expression after the heat shock in control and high CO_2 larvae.

There was a significant interaction in zoea II on day 3 for CCR expression. Subsequent one-way ANOVA showed differences in mean values of treatment groups (table 4) with significantly decreased CCR expression in control larvae after the heat shock of 20°C. CCR was significantly affected by heat shock in the zoea II larvae on day 15 (ANOVA II: $F_{1,13} = 8.653$, $p = 0.011$). Higher CCR expression was found in control larvae at 20°C compared to expression at 10°C.

Heat shock affected expression of COX in zoea I larvae on day 15 (ANOVA II: $F_{1,16} = 16.797$, $p < 0.001$). Expression was significantly lower after the heat shock independent of seawater CO_2 concentration. In zoea II on day 3 and day 15 as well as megalopa larvae one-way ANOVAs showed differences in mean values of treatment groups (table 4). On day 3, COX expression was lower in control zoea II larvae at 20°C. In both larval stages, zoea II on day 15 and megalopa, COX expression decreased significantly in high CO_2 larvae after heat shock.

A significant interaction was detected for the expression of atpA in zoea II on day 3. Subsequent one-way ANOVA showed differences in mean values of treatment groups (table 4) with lower atpA expression in control larvae after heat shock.

CO_2 effect

There was a significant interaction between heat shock and CO_2 concentration detected by the two-way ANOVA for expression of CCR in zoea II on day 3 and of IDH and NAD in zoea II on day 15. Furthermore a significant interaction was detected for gene expression of COX in zoea II larvae on day 3 and day 15.

Although expression of PDH was not significantly affected by seawater CO_2 concentration in zoea II on day 3, Tukey test revealed higher PDH expression in high CO_2 larvae after heat shock compared to control larvae. In the megalopa stage seawater CO_2 concentration influenced PDH expression significantly (ANOVA II: $F_{1,14} = 14.314$, $p = 0.002$). A Tukey test revealed significantly different expression after the heat shock between larvae reared at different CO_2 levels with lower expression in high CO_2 larvae.

One-way ANOVAs showed differences in mean values of the treatment groups for IDH expression in zoea II on day 15 (table 4) with higher expression in high CO_2 larvae compared to control larvae after the heat shock of 20°C. IDH expression was affected by

seawater CO_2 concentration in megalopa (ANOVA II: $F_{1,16}$ = 5.739, p = 0.029) with lower expression in high CO_2 larvae compared to control larvae after the heat shock.

Expression of NAD was significantly affected by seawater CO_2 concentration in zoea I larvae on day 15 (ANOVA II: $F_{1,16}$ = 6.088, p < 0.025). After the heat shock, NAD expression was significantly higher in larvae reared under high CO_2 in comparison to control larvae. Seawater CO_2 concentration also affected NAD expression significantly in zoea II on day 3 (ANOVA II: $F_{1,16}$ = 9.129, p = 0.008) with lower expression at 20°C in high CO_2 larvae. One-way ANOVAs showed differences between mean values of treatment groups in zoea II on day 15 (table 4) with 10-fold higher NAD expression in larvae reared at elevated seawater PCO_2 at 10°C. NAD expression in the megalopa was also affected by seawater CO_2 concentration (ANOVA II: $F_{1,15}$ = 6.367, p = 0.023). At 10°C, larvae reared at elevated seawater PCO_2 showed significantly higher expression.

Expression of succinate dehydrogenase (SDH) was significantly affected by seawater CO_2 concentration in zoea I on day 15 (ANOVA II: $F_{1,16}$ = 6.634, p = 0.020) with higher SDH expression in high CO_2 larvae at 10°C in comparison to control larvae. Expression of SDH was significantly affected by seawater CO_2 concentration in zoea II on day 3 (ANOVA II: $F_{1,16}$ = 10.708, p = 0.005) with higher expression in high CO_2 larvae compared to control larvae independent of temperature.

CCR expression was significant higher in high CO_2 zoea II larvae after the heat shock on day 3. Seawater CO_2 concentration affected expression of CCR in megalopa larvae (ANOVA II: $F_{1,16}$ = 6.138, p = 0.025) with significant lower CCR expression in high CO_2 larvae after heat shock.

Seawater CO_2 concentration affected expression of COX in zoea I larvae on day 15 (ANOVA II: $F_{1,16}$ = 6.127, p = 0.025). Expression was significantly higher in high CO_2 larvae. In zoea II larvae on day 3 and day 15 COX expression was higher at after heat shock in larvae reared at high CO_2 compared to control larvae. On day 15, higher COX expression was found in larvae reared under high CO_2 at 10°C compared to control larvae.

Combined effect of heat shock and CO_2

A Tukey test revealed higher expression of SDH in zoea II on day 3 after the heat shock of 20°C in larvae reared at elevated PCO_2.

Discussion

Determination of the larval thermal tolerance window

In the present study, the concept of oxygen and capacity limited thermal tolerance (Pörtner 2001) was applied to determine the thermal tolerance and putatively synergistic effects of elevated seawater P_{CO_2} in different larval stages of the crustacean *Hyas araneus*. We could show that the three different larval stages of *Hyas araneus* display different upper critical thermal tolerance limits at 25°C in zoea I and zoea II in comparison to 22°C in megalopa larvae. According to Frederich and Pörtner (2000), limited capacities of ventilation and circulation lead to a progressive mismatch between oxygen demand for maintenance and oxygen supply and finally lead to hypoxia and anaerobic metabolism beyond the upper critical limit. Upon further warming, standard metabolic rate and heart beat rate decreases. A corresponding decrease in heart rate and oxygen consumption could also be observed in *Hyas araneus* larvae. In larvae at control seawater CO_2 concentration, oxygen consumption and heart rate were maximal at 25°C in zoea I and zoea II larvae and at 22°C in megalopa larvae and decreased at 28°C in zoea and at 25°C, in megalopa larvae, respectively. The sharp drop in oxygen consumption of the zoea larvae was correlated with ceased maxilliped beating rates. Simultaneously decreasing heart rates of zoea larvae strongly suggest concomitant limitation or onset of failure of both ventilatory and circulatory systems. Different optimum temperature ranges in three larval stages have also been reported for the kelp crab *Taliepus dentatus* with the narrowest window found in the megalopa (Storch et al. 2011). The high sensitivity of megalopae to environmental stressors was also found in previous studies suggesting that this larval stage is a physiologically sensitive bottleneck within the life cycle of decapod crustaceans (Walther et al. 2010; Storch et al. 2011).

The high thermal sensitivity of the megalopa stage might be due to an immature linkage between cardiac performance and metabolic requirements as in early larval fish (Pelster 2002). Referring to the concept of oxygen and capacity limited thermal tolerance (Pörtner 2001; Pörtner and Farrell 2008), a lower Q_{10} for cardiac output than for oxygen consumption in early larval stages would lead to an early oxygen deficit during warming. Q_{10} values for heart rate and oxygen consumption were close to 1.5 between 10°C and 25°C in zoea stages of *Hyas araneus*. In *Hyas araneus* megalopa larvae, the Q_{10} value for oxygen consumption between 10°C and 22°C revealed a strong temperature dependency and was about 5, while the Q_{10} value for heart rate was 1.4 and, thus, much lower. Standard metabolic rates of *Hyas araneus* megalopa were lower than those of the zoea

stages at the rearing temperature of 10°C and showed a stronger increase with warming. However, elevated oxygen demand could not be covered by a concomitant increase in oxygen supply due to limited capacity of the circulatory system. In zoea larvae, the respiratory gas exchange is accomplished by means of diffusion via the whole body surface and they might be less dependent on the supply of oxygen through the heart.

Exposure to elevated seawater P_{CO_2} affected limited the thermal tolerance of zoea stages of *Hyas araneus* and resulted in a downward shift of the upper thermal limits that was less pronounced in zoea I larvae than in zoea II larvae. In zoea I larvae, a decrease in thermal tolerance can be denoted by the higher oxygen consumption at 25°C of larvae under control conditions compared to those incubated under elevated CO_2. The collapse in respiration in high CO_2 zoea I larvae was not accompanied by significantly lower heart rates and maxilliped beating rates. Upon warming increasing heart rates at concomitantly decreasing oxygen consumption rates, could also be seen in the larval stages in two populations of the kelp crab *Taliepus dentatus* and were attributed to a progressive mismatch between oxygen demand and oxygen supply (Storch et al. 2009; Storch et al. 2011). This pattern was more pronounced in the second zoea stage as the drop in oxygen consumption between 22°C and 25°C was more pronounced and accompanied by a decrease in heart rate and ceased maxilliped beating rate at 25°C. In zoea I larvae maxilliped beating rates did not stop until 28°C. Furthermore, mortality of zoea II larvae was twice as high under hypercapnia while mortality of zoea I larvae was only slightly higher under hypercapnia (data not shown). These results indicate higher sensitivities of zoea II larvae towards high seawater P_{CO_2} values. In the study by Walther et al. (2009) on thermal tolerance under elevated seawater P_{CO_2} in adults of *Hyas araneus*, the CO_2 induced rise in Q_{10} values of heart rate has been proposed to be responsible for a narrowed thermal window under high seawater CO_2. Our data are in line with those findings, showing a steep rise in the $Q10$ values of respiration between rearing and critical temperatures in zoea II larvae exposed to high CO_2. Higher tissue oxygen demands with increasing temperature might be compensated for by the higher heart rates (albeit not statistically significant), observed in zoea II larvae under elevated CO_2, indicating a capacity limitation of the circulation system at lower temperature.

Higher oxygen consumption in control compared to larvae under high CO_2 could already be seen at 22°C in megalopa larvae probably as a result of a more pronounced downward shift of the upper thermal limit in the megalopa larvae. This is further reinforced by the highest oxygen consumption rates of megalopa larvae measured at 13°C under CO_2 in

comparison to 22°C in untreated megalopa. However, no significant difference in respiration was detected between different temperatures in high CO_2 megalopa larvae exacerbating statements on their critical temperature. Elevated seawater P_{CO_2} affected oxygen consumption but not heart rate of *Hyas araneus* megalopa, reflecting the limited match between the two processes.

Gene expression patterns

The three physiological parameters (oxygen consumption, heart rate and maxilliped beat rate) were measured in 9-13-day old zoea I and zoea II larvae and can, thus, be aligned with the gene expression data for physiological processes like cellular stress/heat shock response, acid-base regulation and mitochondrial energy metabolism in zoea I and zoea II at day 15. The gene expression data measured on the other developmental days allow for a comparison of CO_2 sensitivities during development.

Cellular stress/heat shock response

We examined gene expression of different heat shock proteins. HSP 70 (1-4) and heat shock protein 90 were highly up-regulated in all three larval stages at any developmental time of *Hyas araneus* upon heat shock at 20°C. However, there was a stronger response to thermal stress in 15 day old than in 3 day old zoea I and zoea II larvae indicating that larvae in the early stage phase might be more tolerant or less responsive to the stress than in the late stage phase. Heat shock proteins help to prevent denaturation of proteins and to refold denatured proteins. The high degree of up-regulation of HSP 70 and HSP 90 in *Hyas araneus* larvae suggests that 20°C is close to the upper thermal limit as also seen in the physiological data of both zoea stages.

There was a synergistic effect of temperature and high seawater P_{CO_2} on HSP70 and HSP90 expression in both zoea stages of *Hyas araneus*, resulting in higher HSP expression at 20°C in larvae reared at high CO_2. This finding was more pronounced in the second zoea stage on day 15 in comparison to the first zoea stage on day 15 and reflects the CO_2 induced downward shift in the upper thermal limit, which was more pronounced in the second zoea stage (see above).

Although there might be a difference between HSP transcription and translation, we assume that the strong increase in HSP expression in *Hyas araneus*, after the heat shock of 20°C, should result in increased protein levels. HSP70 is an ATP-dependent chaperone

and the prevention of heat-induced protein denaturation is a highly ATP-demanding process. Increased expression of HSPs starts at some temperature (*T*on or threshold temperature) above the acclimation temperature and increases until a maximum is reached (*T*peak) and expression starts to drop (*T*off) (Tomanek and Somero 1999). In marine organisms, *T*on was found to be close to the upper pejus temperature at which mortality starts to rise (Anestis et al. 2007), while *T*off was close to the upper critical temperature at which survival was strongly compromised (Tomanek and Somero 1999). There was a correlated decrease of heart rate and HSP expression in three decapod crustaceans (Jost et al. 2012). The heat shock response and threshold temperature for HSP induction is highly plastic responding to acclimation temperature and habitat (Tomanek and Somero 1999; Buckley et al. 2001). Higher threshold temperatures for heat shock protein production as found in warm-acclimatized or summer animals reflect the shifted limits of thermal tolerance and also a trade-off between costs for passive thermal tolerance and costs of thermal denaturation of the protein pool at low HSP levels (Buckley et al. 2001; Hamdoun et al. 2003). The synergistic effect of elevated seawater P_{CO_2} and heat shock with higher HSP expression in high CO_2 zoea larvae could indicate a left shift on the temperature scale, of the three key characteristics of the heat-shock response, *T*on, *T*peak and *T*off, equivalent to the left-shift of the OCLTT thresholds.

This interpretation helps in assessing the findings in *Hyas araneus* megalopa larvae. There was no synergistic effect of elevated seawater P_{CO_2} and heat shock on the gene expression of heat shock proteins in megalopa larvae. This parallel the findings of no effect of CO_2 on heart beat rate and minor changes in oxygen consumption. Previous studies already suggested a stronger response of the megalopa stage of Arctic *Hyas araneus* to thermal stress than to enhanced CO_2 levels (Walther et al. 2010). CO_2 effects also tend to vanish in *Hyas araneus* megalopa larvae from a temperate population around Helgoland (North sea) (Walther et al. 2010). The narrow thermal window of the megalopa indicates distinct stenothermy of this larval stage, which might prevent further narrowing under hypercapnia-exposure or reduce the possibility to detect any small differences in its thermal tolerance. High thermal sensitivity of the megalopa under control conditions is then paralleled by the limited capacity of stress response mechanisms to shift thermal limits or enhance the capacity for passive thermal tolerance, emphasizing the inflexibility or bottleneck characteristics of this larval stage.

Neither exposure to 20°C nor high CO_2 concentration induced an elevated expression of HSP60 in all *Hyas araneus* larval stages. HSP60 is a mitochondrial matrix protein and is

involved in the folding of polypeptides into complex mitochondrial enzymes (Briones et al. 1997). In crustaceans, HSP60 was found to respond to bacterial infections and contaminant exposures (Werner and Nagel 1997; Zhou et al. 2010) and might play a more important role in the immune response than during heat stress. Heat shock protein 26 was the only heat shock protein down regulated at increased temperature. We used CCA (Fig. 4) to visualize the dependence of mRNA expression of the different genes on the three different factors (temperature, CO_2 and stage) and HSP 26 transcript levels were negatively correlated with temperature. These findings are in line with Al-Fageeh et al. (2006) and Colinet et al. (2010) who found that HSP26 was induced by cold in *Drosophila melanogaster* and mammalian cells. This indicates a greater significance of HSP 26 during cold exposure.

Acid-base regulation and mitochondrial energy metabolism

Hyas araneus larvae displaying limited thermal tolerance at elevated seawater P_{CO_2} mirrors findings in adult specimens of spider and edible crabs (Metzger et al. 2007; Walther et al. 2009). This limitation might be attributed to the elevation in CO_2 levels or an incomplete compensation of extracellular acidosis. It is known that elevated seawater P_{CO_2} leads to decreasing extracellular pH in *Hyas araneus* adults (Zittier et al. 2012), which might cause metabolic depression in tissues and cells as found in invertebrates and fish (Reipschläger and Pörtner 1996, Langenbuch and Pörtner 2003). Metabolic depression might concomitantly decrease the capacity to increase aerobic energy turnover at increasing temperatures. In our study, gene expression of acid-base related transporters and enzymes as well as enzymes from mitochondrial pathways were examined to determine whether or not acid-base regulation and / or metabolism respond to elevated seawater P_{CO_2} at a transcriptomic level.

Acid-base regulation under elevated CO_2 mainly involves active ion transporters like H^+-ATPases or transporters (sodium potassium chloride cotransporter NKCC; sodium bicarbonate co-transporter NBC), depending on the ion gradient maintained by sodium potassium ATPase (NaK). Carbonic anhydrase (CA) facilitates the formation of bicarbonate (Henry and Cameron 1983; Pörtner 2008; Melzner et al. 2009). Transcript sequences related to ion and acid-base regulation and responding to thermal stress (CA, NaK, NKCC), were down regulated in *Hyas araneus* larvae from both CO_2 treatments, reflecting thermal compensation as higher enzyme activities at higher temperatures might involve reduced gene expression. This is in line with findings by Edge et al. (2005) on coral gene expression associated with stressful temperature conditions. Coral carbonic anhydrase

also showed a decrease in expression at elevated temperatures. Conversely, cold acclimated fish responded by enhancing Na^+K^+-ATPase gene expression (Metz et al. 2003). Thermal compensation predominates as in all larval stages, *Hyas araneus* showed no strong response in the expression of transporters and enzymes to high seawater CO_2 levels. Under elevated CO_2 levels an up-regulation of proteins (CA, NBC, NKCC) related to acid-base regulation was mainly seen in 3 day old zoea II and megalopa larvae indicating higher compensatory capacities to maintain acid-base homeostasis.

Metabolic depression during hypoxia was indicated by the repression of genes in the citric acid cycle and in the electron transport system in gills of adult zebrafish (van der Meer et al. 2005). Gene expression of various genes from the citric acid cycle and the electron transport system gave no indication of a CO_2 induced metabolic depression in *Hyas araneus* larvae. The down-regulation of genes was again mainly associated with an increase in temperature. This was supported by the CCA (Fig. 4) where the negative correlation of most of the mitochondrial genes indicates reduced expression at elevated temperature and thereby, the tyoical effects of warm acclimation in eurytherms (Pörtner et al. 2000b; Lannig et al. 2005).

The majority of genes from mitochondrial metabolic pathways responding to CO_2 stress were up-regulated (Tab. 5), although the CCA reflects a stronger impact of high temperature. NADH dehydrogenase was the only mitochondrial gene in *Hyas araneus* larvae, which showed a positive correlation with seawater P_{CO_2} in the canonical correspondence analysis (Fig. 4). Up-regulation of enzymes of the electron transport system and the citric acid cycle in larvae reared at elevated seawater P_{CO_2} could point to a compensation of elevated demand on mitochondrial energy or a compensation for reduced mitochondrial capacities under elevated CO_2 levels. The latter seems to be the case. An increased energy demand in high CO_2 larvae should be reflected in higher metabolic rates, which was not the case in *Hyas araneus*. It seems that a higher number of enzymes was necessary for the maintenance of standard metabolism in the high CO_2 treatment. This could in fact be due to lower enzyme activities at elevated seawater P_{CO_2}. Strobel et al. (2012) reported lower cytochrome c oxidase activity in the Antarctic fish *Notothenia rossii* exposed to seawater CO_2 levels of 2000 µatm. The phenomenon that elevated atmospheric CO_2 inhibits mitochondrial respiration and respiratory enzymes, has long been recognized in plants (Gonzàles-Meler et al. 1996; Drake et al. 1999). Elevated atmospheric CO_2 inhibited the oxidation of succinate and external NADH and cytochrome c oxidase and succinate dehydrogenase activity (Gonzàles-Meler et al. 1996). Drake et al.

(1999) discussed that bicarbonate inhibits plant cytochrome c oxidase and succinate dehydrogenase by binding to the enzyme (Miller and Evans 1956; Zeylemaker et al. 1970). Bicarbonate also inhibited citrate synthase in mouse liver mitochondria (Simpson, 1967) and activates adenylyl cyclase, which produces the second messenger cAMP, which is involved in enzyme regulation by phosphorylation and also transcription factor regulation (Acin-Perez 2009; Tresguerres et al. 2011). As bicarbonate levels rise in parallel to rising CO_2 levels in intracellular as well as extracellular compartments in marine organisms (Pane and Barry 2007; Strobel et al. 2012; Zittier et al. 2012), it might inhibit enzymes of the mitochondrial metabolic pathways. An up-regulation of these enzymes, as we found in *Hyas araneus* larvae, could be a compensatory measure to maintain standard metabolic rates and aerobic scope at high seawater CO_2 levels.

Interestingly, these regulation mechanisms in ion transport and metabolism were mainly seen in 3 day old zoea II accompanied by a lower heat shock response than in 15 day old zoea II. More thermally tolerant 3 day old zoea II when still within their thermal window are able to compensate for high seawater CO_2 levels by up and down regulation of enzymes supporting cellular homeostasis and/or metabolic pathway fluxes. In contrast, less thermally tolerant 15 day old zoea II are already forced to protect their proteins by an increased HSP response for passive survival and are beyond the temperature where regulating mechanisms can maintain cellular functioning.

Conclusion

Our findings reveal differences in thermal tolerance between the three larval stages of the spider crab *Hyas araneus* with the narrowest window found in the megalopa. The high thermal sensitivity of the megalopa stage might be due to an insufficient match between cardiac performance and metabolic requirements leading to an early mismatch between oxygen demand and oxygen supply during acute warming. Exposure to elevated seawater P_{CO_2} narrowed the thermal tolerance window of zoea larvae with a breakdown in respiration and heart rate at a lower temperature. The distinct stenothermy of the megalopa stage might prevent further limitation of thermal tolerance during hypercapnic exposure.

In previous studies, effects of elevated seawater P_{CO_2} on thermal tolerance of marine organisms focused on whole animal performance, showing synergistic effects of high CO_2 and high temperature (Metzger et al. 2007; Walther et al. 2009; Zittier et al. 2012). However, our knowledge of mechanisms affected by both factors and shaping sensitivities

of an organism to ocean acidification and warming are far from being complete and further studies are necessary. In the present mechanistic study, we were able to reveal mechanisms at the molecular level that were affected by high temperature, high CO_2 and the combined action of both factors. In different larval stages of the spider crab *Hyas araneus*, we found a strong CO_2 effect with an up-regulation of genes involved in oxidative phosphorylation, indicating compensation of enzyme activities limited by bicarbonate inhibition. A strong increase in HSP expression in zoea stages of *Hyas araneus* under heat stress and CO_2 reflects an exacerbation of thermal stress and the capacity to adjust passive tolerance at the edges of the thermal window. Our study underlines the importance of integrative approaches to link molecular and cellular to whole organism and ecological responses to understand the biological consequences of ocean acidification and warming.

Acknowledgements

Financial support was provided by Federal Ministry of Education and Research (BMBF), within phase I of the BIOACID research programme (FKZ 03F0608B, subproject 2.2.1 PI D. Storch). We thank the scientific T. Sandersfeld for technical assistance.

References

Acin-Perez R, Salazar E, Kamenetsky M, Buck J, Levin LR and Manfredi G (2009) Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation. Cell metab 9 (3): 265-276

Al-Fageeh MB, Marchant RJ, Carden MJ and Smales CM (2006) The cold-shock response in cultured mammalian cells: harnessing the response for the improvement of recombinant protein production. Biotechnol Bioeng 93 (5): 829-835

Anestis A, Lazou A, Pörtner HO and Michaelidis B (2007) Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. Am J Physiol Regul Integr Comp Physiol 293: R911–R921

Briones P, Vilaseca MA, Ribes A, Vernet A, Lluch M, Cusi V, Huckriede A and Agsteribbe E (1997) A new case of multiple mitochondrial enzyme deficiencies with decreased amount of heat shock protein 60. J Inherit Metab Dis 20: 569-577

Buckley BA, Owen ME and Hofmann GE (2001) Adjusting the thermostat: the threshold

induction temperature for the heatshock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. J Exp Biol 204: 3571–3579

Caldeira K and Wickett ME (2005) Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J Geophys Res-Oceans 110, C09S04

Chapman R, Mancia A, Beal M, Veloso A, Rathburn C, Blair A, Holland AF, Warr GW, Didinato G, Sokolova IM, Wirth EF, Duffy E and Sanger D (2011) The transcriptomic responses of the eastern oyster, *Crassostrea virginica*, to environmental conditions. Mol Ecol 20 (7): 1431-1449

Christiansen ME (1969) Crustacea Decapoda Brachyura. Marine invertebrates of Scandinavia, vol 2. Universitetsforlaget, Oslo

Colinet H, Lee SF and Hoffmann A (2010) Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. FEBS J 277 (1): 174-185

Drake BG, Azcon-Bieto J, Berry J, Bunce J, Dijkstra P, Farrar J, Gifford M, Gonzalez-Meler, Koch G, Lambers H, Siedow J and Wullschleger S (1999) Does elevated atmospheric CO_2 concentration inhibit mitochondrial respiration in green plants? Plant cell environ 22: 649–657

Edge SE, Morgan MB, Gleason DF and Snell TW (2005) Development of a coral cDNA array to examine gene expression profiles in *Montastraea faveolata* exposed to environmental stress. Mar Pollut Bull 51: 507–523

Frederich M and Pörtner HO (2000) Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*. Am J Physiol Regul Integr Comp Physiol 279, R1531-R1538

Gonzàlez-Meler MA, Ribas-Carbó M, Siedow JN and Drake BG (1996) Direct inhibition of plant mitochondrial respiration by elevated CO_2. Plant Physiol 112: 1349-1355

Hamdoun AM, Cheney DP and Cherr GN (2003) Plasticity of HSP70 and HSP70 gene expression in the Pacific Oyster (*Crassostrea gigas*): Implications for thermal limits and induction of thermal tolerance. Biol Bull 205: 160–169

Henry RP and Cameron JN (1983) The role of carbonic-anhydrase in respiration, ion regulation and acid-base-balance in the aquatic crab *Callinectes-sapidus* and the terrestrial crab *Gecarcinus lateralis*. J Exp Biol 103: 205–223

IPCC (2007) Climate Change 2007: the physical science basis. Summary for policymakers. Contribution of working group I to the fourth assessment report. The intergovernmental panel on climate change. Mar Ecol Prog Ser 444: 263–274

Jost JA, Podolski SM and Frederich M (2012) Enhancing thermal tolerance by eliminating the pejus range: a comparative study with three decapod crustaceans. Mar Ecol-Prog Ser 444: 263-274

Kieffer JD, Currie S and Tufts BL (1994) Effects of environmental temperature on the metabolic and acid-base responses of rainbow trout to exhaustive exercise. J Exp Biol 194: 299-317

Kregel KC (2002) Invited Review: Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol 92: 2177–2186

Kurihara H (2008) Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. Mar Ecol Prog Ser 373: 275–284

Langenbuch M and Pörtner HO (2003) Energy budget of hepatocytes from Antarctic fish (*Pachycara brachycephalum* and *Lepidonotothen kempi*) as a function of ambient CO_2: pH-dependent limitations of cellular protein biosynthese? J Exp Biol 206: 3895-3903

Lannig G, Storch D and Pörtner HO (2005) Aerobic mitochondrial capacities in Antarctic and temperate eelpout (Zoarcidae) subjected to warm versus cold acclimation. Polar Biol 28: 575–584

Melzner F, Gutowska MA, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M and Pörtner HO (2009) Physiological basis for high CO_2 tolerance in marine ectothermic

animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6: 2313–2331

Metz JR, van den Burg EH, Wendelaar Bonga SE and Flik G (2003) Regulation of branchial Na+/K+-ATPase in common carp *Cyprinus carpio* L. acclimated to different temperatures. J Exp Biol 206: 2273-2280

Metzger R, Sartoris FJ, Langenbuch M and Pörtner HO (2007) Influence of elevated CO_2 concentrations on thermal tolerance of the edible crab *Cancer pagurus*. J Therm Biol 32: 144-151

Miller GW and Evans HJ (1956) Inhibition of plant cytochrome oxidase by bicarbonate. Nature 178: 974-976

O'Donnell MJ, Hammond LM and Hofmann GE (2009) Predicted impact of ocean acidification on a marine invertebrate: elevated CO2 alters response to thermal stress in sea urchin larvae. Mar Biol 156: 439-446

Pane EF and Barry JP (2007) Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. Mar Ecol Prog Ser 334: 1-9

Pelster B (2002) Developmental plasticity in the cardiovascular system of fish, with special reference to the zebra fish. Comp Biochem Physiol 133 A: 547–553

Pörtner HO (2001) Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. Naturwissenschaften 88: 137–146

Pörtner HO (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. Mar Ecol Prog Ser 373: 203–217

Pörtner HO (2010) Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J Exp Biol 213: 881-893

Pörtner HO and Zielinski S (1998) Environmental constraints and the physiology of performance in squids. In: Payne AIL, Lipinski MR, Clarke MR, Roeleveld MAC (eds) Cephalopod biodiversity, ecology and evolution. S Afr J Mar Sci 20:207–221

Pörtner HO and Farrell AP (2008) Physiology and climate change. Science 322: 690-692

Pörtner HO, Reipschlager A and Heisler N (1998) Acid–base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. J Exp Biol 201:43–55

Pörtner HO, Bock C and Reipschläger A (2000a) Modulation of the cost of pHi regulation during metabolic depression: A 31P-NMR study in invertebrate (*Sipunculus nudus*) isolated muscle. J Exp Biol 203: 2417-2428

Pörtner HO, Van Dijk PLM, Hardewig I and Sommer A (2000b) Levels of metabolic cold adaptation: tradeoffs in eurythermal and stenothermal ectotherms. In: Antarctic Ecosystems: Models for a Wider Understanding, Davison W and Williams CW (eds), Christchurch, New Zealand: Caxton

Pörtner HO, Langenbuch M and Reipschläger A (2004) Biological Impact of elevated ocean CO_2 concentrations: Lessons from animal physiology and earth history. J Oceanogr 60: 705-718

Pörtner HO, Langenbuch M and Michaelidis B (2005) Synergistic effects of temperature extremes, hypoxia, and increases in CO_2 on marine animals: From Earth history to global change. J Geophys Res 110, C09S10

Reipschläger A and Pörtner HO (1996) Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. J Exp Biol 199: 1801–1807

Schalkhausser B, Bock C, Stemmer K, Brey T, Pörtner HO and Lannig G (2012) Impact of ocean acidification on escape performance of the king scallop, *Pecten maximus*, from Norway. Mar Biol DOI 10.1007/s00227-012-2057-8

Simpson DP (1967) Regulation of renal citrate metabolism by bicarbonate ion and pH: observations in tissue slices and mitochondria. J Clin Invest 46(2): 225

Storch D, Santelices P, Barria J, Cabeza K, Pörtner HO and Fernández M (2009) Thermal

tolerance of crustacean larvae (zoea I) in two different populations of the kelp crab *Taliepus dentatus* (Milne-Edwards) J Exp Biol 212: 1371-1376

Storch D, Fernández M, Navarrete SA and Pörtner HO (2011) Thermal tolerance of larval stages of the Chilean kelp crab *Taliepus dentatus.* Mar Ecol Prog Ser 429: 157–167

Strobel A, Bennecke S, Leo E, Mintenbeck K, Pörtner HO and Mark FC (2012) Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2. Front Zool 9 (28) doi:10.1186/1742-9994-9-28

Stumpp M, Wren J, Melzner F, Thorndyke MC and Dupont ST (2011a) CO_2 induced seawater acidification impacts sea urchin larval development I: Elevated metabolic rates decrease scope for growth and induce developmental delay. Comp Biochem Phys A 160: 331-340

Stumpp M, Dupont S, Thorndyke MC and Melzner F (2011b) CO_2 induced seawater acidification impacts sea urchin larval development II: Gene expression patterns in pluteus larvae. Comp Biochem Phys A 160: 320-330

Todgham AE and Hofmann GE (2009) Transcriptomic response of sea urchin larvae *Strongylocentrotus purpuratus* to CO_2-driven seawater acidification. J Exp Biol 212: 2579-2594

Tomanek L and Somero GN (1999) Evolutionary and acclimation-induced variation in the heat-shock response of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. J Exp Biol 202: 2925-2936

Tresguerres M, Levin LR and Buck J (2011) Intracellular cAMP signaling by soluble adenylyl cyclase. Kidney Int 79, 1277-1288

van der Meer DLM, van den Thillart GEEJM, Witte F, de Bakker MAG, Besser J, Richardson MK, Spaink, HP, Leito JTD and Bagowski CP (2005) Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. Am J Physiol Regul Integr Comp Physiol 289: R1512–R1519

Walther K, Sartoris FJ, Bock C and Pörtner HO (2009) Impact of anthropogenic ocean acidification on thermal tolerance of the spider crab *Hyas araneus*. Biogeosciences 6: 2207-2215

Walther K, Anger K and Pörtner HO (2010) Effects of ocean acidification and warming on the larval development of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°N). Mar Ecol Prog Ser 417: 159-170

Werner I and Nagel R (1997) Stress proteins HSP60 and HSP70 in three species of amphipods exposed to cadmium, diazinon, dieldrin and fluoranthene. Environ Toxicol Chem 16 (11): 2393-2403

Wittmann AC, Schröer M, Bock C, Steeger HU, Paul RJ and Pörtner HO (2008) Indicators of oxygen- and capacity-limited thermal tolerance in the lugworm *Arenicola marina.* Clim Res 37: 227–240

Zeylemaker WP, Klaasse ADM, Slater EC and Veeger C (1970) Studies on succinate dehydrogenase VI. Inhibition by monocarboxylic acids. Biochim Biophys Acta 198: 415-422

Zhou J, Wang WN, He WY, Zheng Y, Wang L, Xin Y, Liu Y and Wang AL (2010) Expression of HSP60 and HSP70 in white shrimp, *Litopenaeus vannamei* in response to bacterial challenge. J Invertebr Pathol 103 (3): 170-178

Zittier, ZMC, Hirse T and Pörtner HO (2012) The synergistic effects of increasing temperature and CO_2 levels on activity capacity and acid–base balance in the spider crab, *Hyas araneus*. Mar Biol DOI 10.1007/s00227-012-2073-8

Tables

Table 1 Seawater parameters measured during incubation. Values are given in mean ± SD. N = 5 NBS: National Bureau of Standards; DIC: dissolved inorganic carbon; PCO_2: partial pressure of CO_2.

Incubation	**Temperature (C°)**	**pH_{NBS}**	**DIC (µmol/kg)**	**PCO_2 (µatm)**	**Salinity (PSU)**
Control	10.0±0.5	8.04±0.03	2277±25	428±35	31.8±0.3
High CO_2	10.0±0.5	7.18±0.03	2473±67	3390±169	31.8±0.3

Table 2 Primer of 21 genes used for RT-qPCR including primer ID, protein description, primer sequence and primer efficiency.

Primer ID	Protein description	Primer Sequence	Primer efficiency
Cellular stress/heat shock response			
HSP 26	Heat shock protein 26	F_AGGCAAGAGGCCGACAGA B_AAGCGGCGGTTGAAACG	1.98
HSP 60	Heat shock protein 60	F_GCCAACGGCACCTTCGT B_CCTTGGTGGGATCGATGATT	1.99
HSP 70_1	Heat shock protein 70	F_AGCACTTCGTCGGCTAAGGA B_CCTGGGCAGATGATGAAAGAG	2.01
HSP 70_2	Heat shock protein 70	F_GTGTGGGCGTGTTCAAGAATG B_CGGTTGCCCTGGTCGTT	2.03
HSP 70_3	Heat shock protein 70	F_CAACGTGCTCATCTTCGATCTG B_CTCGATGGTCAGGATGGATAC A	1.98
HSP 70_4	Heat shock protein 70	F_CCAACATGTCGGGAGAGATGA B_CATGAGCGTTCCCCTAGGAA	2.00
HSP 90	Heat shock protein 90	F_GACACATCCACCATGGGATACA B_TGCTGTGGTCTGGGTTGATC	2.03
Acid-base regulation			
CA	Carbonic anhydrase	F_TACGTGTCGGCCGATAGCA B_AAAGTCCGACCCGCTTCAC	1.92

NBC	Sodium bicarbonate cotransporter	F_CCGCCGTCATTGTCAACAG B_TGGTATCCGCCACCCTTCT	2.01
NKA	Sodium potassium ATPase	F_CCCCGAGAGGATCCTTGAAC B_AGGCTTCTCCTCGCCATTC	2.06
NKCC	Sodium potassium chloride cotransporter	F_GGGCAAGGACATCAGAAAGG B_TCTACTTCACGGCGGAGCTT	1.96
NHE	Sodium hydrogen exchanger	F_GCGGAGACCTGCTGGCTAT B_CGACTTGGCTAACACGTATTG G	1.99
VA	V1-ATPase	F_CACCCCATCCCCGATCTC B_CTGCCGCTCCACGTAGATTT	1.96
Mitochondrial energy metabolism			
atpA	ATP synthase	F_GGTGAATACTTCCGCGACAAC B_TGCTTGGACAGATCGTCGTAG A	1.96
CCR	Cytochrome C reductase	F_GATCAGACCCAGACCAGTCCTT B_CATAGCGCCGGAGGTGTT	1.99
COX	Cytochrome C oxidase	F_CGCTGCAGATGTTATTCACTCAT B_TCCAGGGATAGCATCAGCTTTT	1.99
IDH	Isocitrate dehydrogenase	F_TGGCTCAAAAGAGGACCTATGC B_CCACCACCGGGTTCTTCAC	1.95
NDH	NADH dehydrogenase	F_CCCATAATTAACATCTCGGCAAA B_CTGCCCACATTGATTTAGCTTTT	1.98
SDH	Succinate dehydrogenase	F_CTCCGAGGAGAGGCTCAAGA B_GGTGTGGCAGCGGTATACG	2.02
PD	Pyruvate dehydrogenase	F_CTGGACGAGGAGACCATCGT B_TCCACCGTCACCAGGTTGT	2.02
Potential housekeeping			
Tub	Tubulin	F_GAGGACGCGGCCAACA B_GACAATTTCCTTGCCGATGGT	2.03

Table 3 Results of ANOVAs conducted to investigate effects of CO_2 and temperature on oxygen consumption (Fig. 1A-C), heart rate (Fig. 2A-C) and maxilliped beat rate (Fig. 3 A&B) of *Hyas araneus* zoea and megalopa larvae.

Test	**Stage**	**Response variable**	**CO_2 effect**			**Temperature effect**			**Interaction**		
			F	**d.f**	***p***	**F**	**d.f**	***p***	**F**	**d.f**	***p***
Two-way ANOVA	Zoea I	Oxygen consumption	1	0.419	0.520	6	33.846	**< 0.001**	6	1.562	0170
Two-way ANOVA	Zoea II	Oxygen consumption	1	5.717	**0.020**	6	52.898	**< 0.001**	6	12.363	**< 0.001**
One-way ANOVA	Zoea II	Oxygen consumption – 440 µatm				6	56.391	**< 0.001**			
Kruskal – Wallis one way Analysis of Ranks	Zoea II	Oxygen consumption – 3300 µatm				6		**< 0.001**			
Two-way ANOVA	Megalopa	Oxygen consumption	1	2.461	0.122	6	6.332	**< 0.001**	6	3.927	**0.002**
One-way ANOVA	Megalopa	Oxygen consumption - 440 µatm				6	10.469	**< 0.001**			
One-way ANOVA	Megalopa	Oxygen consumption - 3300 µatm				6	2.271	0.064			
Two-way ANOVA	Zoea I	Heart rate	1	0.0124	0.912	6	17.865	**< 0.001**	6	0.642	0.696
Two-way ANOVA	Zoea II	Heart rate	1	0.0587	0.810	6	25.097	**< 0.001**	6	10.855	**< 0.001**
One-way ANOVA	Zoea II	Heart rate – 440 µatm				6	14.037	**< 0.001**			
One-way ANOVA	Zoea II	Heart rate - 3300 µatm				6	27.783	**< 0.001**			
Two-way ANOVA	Megalopa	Heart rate	1	0.703	0.406	6	18.625	**< 0.001**	6	0.337	0.914
Two-way ANOVA	Megalopa	Maxilliped beat rate	1	1.612	0.210	6	4.625	**< 0.001**	6	0.410	0.868
Two-way ANOVA	Zoea II	Maxilliped beat rate	1	1.520	0.225	6	17.503	**< 0.001**	6	1.440	0.223

Table 4 Results of one-way ANOVAs conducted to investigate differences in gene expression of treatment groups of *Hyas araneus* zoea and megalopa larvae (see arrows in table 5).

Gene	**Life stage/day**	**ANOVA**	
		F	***p***
Cellular stress/heat shock response			
HSP 70_1	Zoea II/3	40,408	**<0,001**
HSP 70_1	Zoea II/15	55,043	**<0,001**
HSP 70_4	Zoea II/3	19,764	**<0,001**
HSP 70_4	Megalopa	11,588	**<0,001**
HSP 90	Megalopa		**0,001**
HSP 90	Zoea II/15	83,712	**<0,001**
Acid-base regulation			
CA	Zoea II/15	14,033	**<0,001**
CA	Megalopa	4,382	**0,020**
NBC	Zoea II/15		0,086
NAD	Zoea II/15	21,564	**<0,001**
Mitochondrial energy metabolism			
Cyt oxi	Zoea II/3	16,812	**<0,001**
Cyt oxi	Zoea II/15	11,352	**<0,001**
Cyt oxi	Megalopa		**0,016**
ATP syn	Zoea II/3	10,379	**<0,001**
Cyt red	Zoea II/3	6,714	**0,004**
NKCC	Megalopa	9,537	**<0,001**
Cyt red	Zoea II/3	6,714	**0,004**
ISD	Zoea II/15	7,608	**0,003**

Table 5 Gene expression (quantities) in zoea and megalopa larvae of *Hyas araneus* at different time points in development. Larvae were reared at control PCO_2 (C) and high PCO_2 (CO_2) at control temperature (10°C) or exposed to a heat shock for 5h at 20°C. Arrow direction indicates significantly higher (upwards) or lower (downwards) gene expression. Black arrows: CO_2 effect at the same temperature (10°C or 20°C). White arrows: heat shock effect in control or high CO_2 larvae. White/Black arrows in one direction indicate a combined effect of CO_2 and heat shock.

Gene	Zoea I day 0		Zoea I day 15				Zoea II day 3				Zoea II day 15				Megalopa day 3			
	C 10°C	C 20°C	C 10°C	CO_2 10°C	C 20°C	CO_2 20°C	C 10°C	CO_2 10°C	C 20°C	CO_2 20°C	C 10°C	CO_2 10°C	C 20°C	CO_2 20°C	C 10°C	CO_2 10°C	C 20°C	CO_2 20°C
Cellular stress/heat shock response																		
HSP 70_1	0.39± 0.02	1.85± 0.29	0.36± 0.03	0.42± 0.06	1.72± 0.10	2.09± 0.20	0.77± 0.07	0.66± 0.10	1.31± 0.08	1.76± 0.07	0.27± 0.03	0.25± 0.01	1.00± 0.16	1.51± 0.11	0.19± 0.01	0.24± 0.03	1.04± 0.10	0.94± 0.21
HSP 70_2	0.92± 0.14	2.14± 0.38	0.84± 0.07	1.01± 0.11	1.97± 0.15	2.19± 0.08	0.47± 0.01	0.40± 0.02	1.11± 0.13	0.88± 0.06	0.69± 0.05	0.83± 0.04	1.46± 0.14	1.91± 0.11	0.71± 0.05	0.73± 0.04	1.59± 0.08	1.40± 0.09
HSP 70_3	0.39± 0.10	1.19± 0.19	0.56± 0.07	1.03± 0.25	1.55± 0.25	1.87± 0.29	0.85± 0.03	0.77± 0.10	1.18± 0.13	1.08± 0.07	0.64± 0.12	0.71± 0.04	1.15± 0.04	1.56± 0.12	0.44± 0.05	0.64± 0.03	1.05± 0.17	1.17± 0.16
HSP 70_4	0.94± 0.27	2.17± 0.41	0.84± 0.05	1.56± 0.24	1.87± 0.17	2.43± 0.19	0.75± 0.04	0.81± 0.10	1.23± 0.15	1.81± 0.11	0.69± 0.04	0.88± 0.07	1.31± 0.03	1.58± 0.13	0.51± 0.03	0.89± 0.10	1.71± 0.23	1.41± 0.18
HSP 90	1.01± 0.32	1.82± 0.29	0.94± 0.09	1.10± 0.16	1.90± 0.22	2.13± 0.19	1.26± 0.05	1.11± 0.22	1.41± 0.16	1.84± 0.04	0.81± 0.04	0.69± 0.03	1.19± 0.04	1.63± 0.07	0.55± 0.02	0.66± 0.07	1.89± 0.19	1.40± 0.11
HSP 26	1.18± 0.24	2.16± 0.37	1.08± 0.18	2.00± 0.29	0.97± 0.22	1,48± 0.36	1.47± 0.09	1.68± 0.37	0.80± 0.07	1.16± 0.09	2.23± 0.10	2.03± 0.13	1.69± 0.08	1.61± 0.12	1.28± 0.08	1.57± 0.05	1.22± 0.13	1.28± 0.09
HSP 60	1.40± 0.10	1.98± 0.43	1.56± 0.12	2.08± 0.43	1.94± 0.05	2.10± 0.18	1.32± 0.15	1.25± 0.24	0.90± 0.08	0.99± 0.18	1.11± 0.13	1.53± 0.12	1.24± 0.02	1.61± 0.18	1.08± 0.05	0.91± 0.08	1.40± 0.31	1.01± 0.12
Acid-base regulation																		
CA	1.59± 0.26	2.06± 0.39	1.50± 0.14	1.78± 0.24	1.52± 0.18	1.65± 0.11	1.31± 0.10	1.58± 0.07	1.10± 0.08	1.23± 0.06	2.20± 0.09	1.55± 0.08	1.74± 0.08	1.62± 0.06	1.35± 0.06	0.99± 0.10	1.26± 0.07	1.24± 0.05
NaK	1.30± 0.31	1.74± 0.34	1.64± 0.09	1.51± 0.25	1.55± 0.15	1.26± 0.10	1.35± 0.07	1.46± 0.09	0.96± 0.05	0.95± 0.03	1.79± 0.10	1.34± 0.14	1.59± 0.11	1.43± 0.12	1.27± 0.09	1.32± 0.08	1.16± 0.10	1.17± 0.06
NBC	1.24± 0.30	2.04± 0.37	1.82± 0.08	1.44± 0.20	1.87± 0.15	1.24± 0.10	1.25± 0.09	1.65± 0.04	1.15± 0.05	1.51± 0.04	1.90± 0.34	089± 0.05	1.02± 0.18	0.88± 0.04	1.29± 0.05	1.49± 0.06	1.33± 0.03	1.37± 0.04
NKCC	0.92± 0.28	1.77± 0.44	1.27± 0.07	1.97± 0.43	1.56± 0.12	2.05± 0.21	1.19± 0.15	1.08± 0.33	0.93± 0.16	1.02± 0.20	1.31± 0.15	1.50± 0.12	1.28± 0.02	1.51± 0.11	1.18± 0.08	1.46± 0.05	1.10± 0.08	0.96± 0.00
Mitochondrial energy metabolism																		
PDH	1.29± 0.29	1.33± 0.29	1.76± 0.09	1.76± 0.35	1.20± 0.23	1.47± 0.13	1.51± 0.04	1.49± 0.12	0.98± 0.14	1.35± 0.09	1.74± 0.18	1.76± 0.08	1.78± 0.18	1.65± 0.10	1.28± 0.08	1.05± 0.01	1.26± 0.11	0.91± 0.04
IDH	1.52± 0.20	2.13± 0.37	1.70± 0.04	2.75± 0.37	1.79± 0.09	2.25± 0.07	2.02± 0.12	1.89± 0.07	1.83± 0.15	1.66± 0.06	1.45± 0.10	1.53± 0.05	1.17± 0.06	1.64± 0.05	1.45± 0.08	1.39± 0.04	1.54± 0.06	1.29± 0.07
NAD	1.19± 0.15	1.34± 0.16	0.98± 0.14	1.34± 0.26	1.05± 0.05	1.83± 0.35	0.83± 0.02	0.48± 0.11	0.73± 0.18	0.37± 0.09	0.20± 0.04	1.80± 0.15	1.17± 0.08	1.69± 0.21	0.82± 0.27	1.46± 0.08	0.98± 0.11	1.26± 0.19
SDH	1.55± 0.15	2.07± 0.41	1.69± 0.10	2.31± 0.29	1.84± 0.12	2.14± 0.14	1.37± 0.08	1.58± 0.06	1.11± 0.09	1.34± 0.03	1.77± 0.15	1.54± 0.05	1.60± 0.06	1.60± 0.06	1.29± 0.02	1.35± 0.09	1.38± 0.04	1.31± 0.07
CCR	1.27± 0.28	2.04± 0.37	1.49± 0.15	2.12± 0.32	1.68± 0.13	1.72± 0.11	1.43± 0.12	1.36± 0.12	0.99± 0.07	1.56± 0.05	1.28± 0.12	1.46± 0.05	1.65± 0.14	1.64± 0.08	1.43± 0.11	1.37± 0.08	1.57± 0.12	1.16± 0.05
COX	1.38± 0.27	2.07± 0.38	1.73± 0.11	2.47± 0.45	0.85± 0.13	1.33± 0.10	1.38± 0.12	1.18± 0.08	0.53± 0.04	1.00± 0.10	1.37± 0.21	2.36± 0.09	1.60± 0.19	1.56± 0.07	1.16± 0.15	1.53± 0.14	1.00± 0.09	0.79± 0.04
atpA	1.44± 0.27	2.10± 0.28	1.81± 0.03	2.68± 0.38	2.72± 0.04	2.09± 0.09	1.49± 0.06	1.49± 0.04	1.14± 0.05	1.56± 0.08	1.93± 0.13	2.12± 0.09	1.89± 0.14	1.79± 0.05	1.62± 0.06	1.61± 0.08	1.65± 0.06	1.52± 0.11

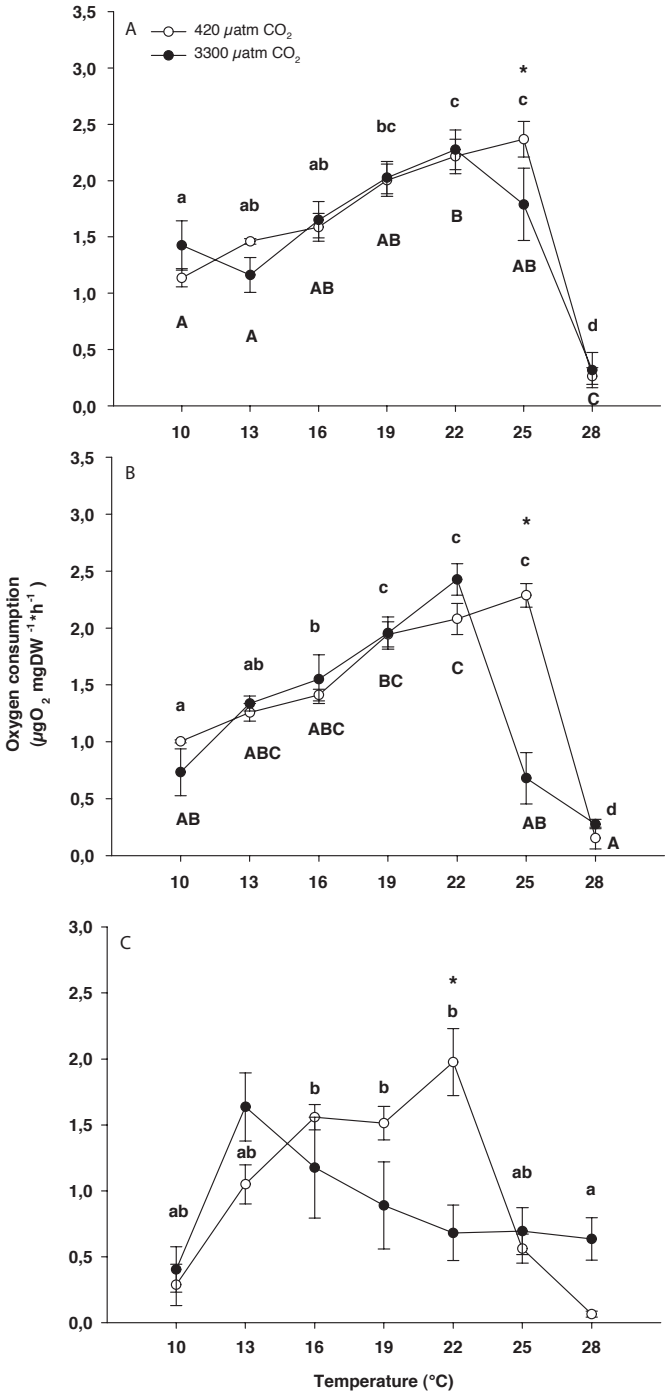


Fig. 1 Temperature dependent oxygen consumption of zoea I (A), zoea II (B) and megalopa larvae (C) of *Hyas araneus* reared at two different seawater P_{CO_2} (open circle: 420 µatm CO_2; closed circle: 3300 µatm CO_2; Mean±SE). Asterisks indicate significant differences between treatments at the same experimental temperature. Different letters indicate significant differences between temperatures within one treatment (lowercase letters: 420 µatm CO_2; uppercase letters: 3300 µatm CO_2).

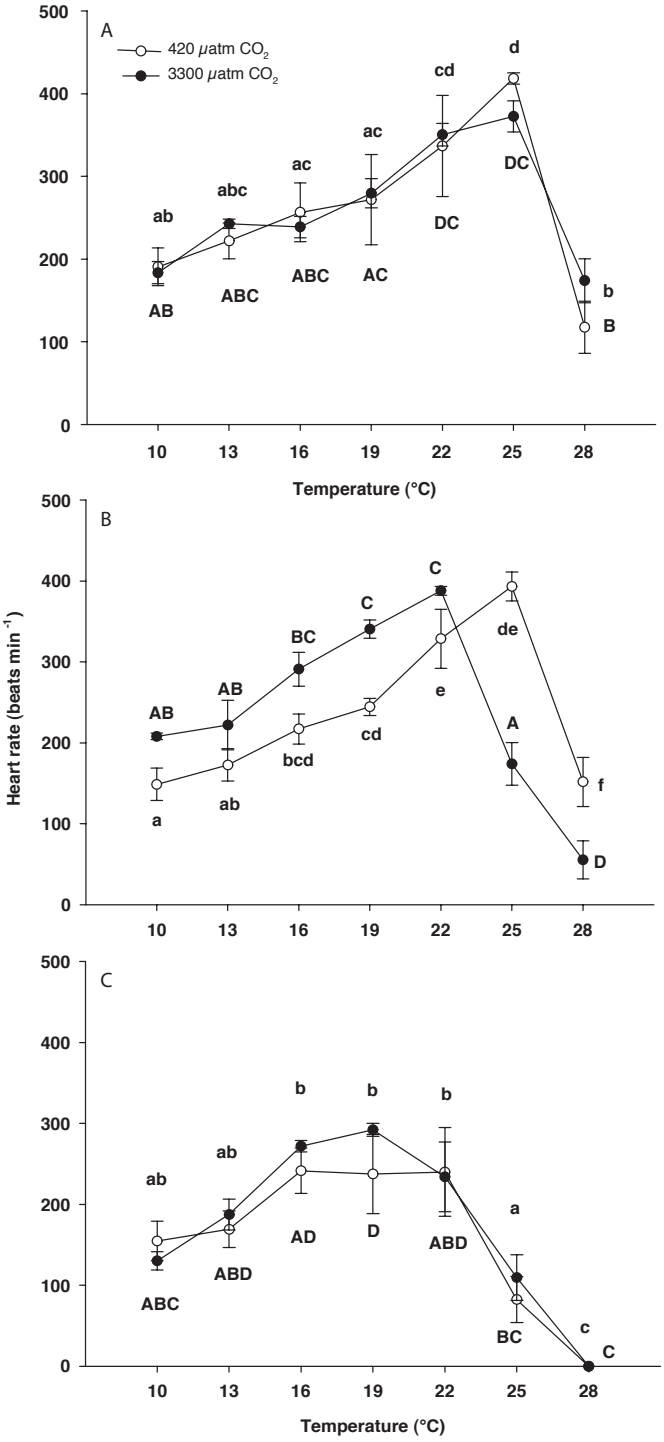


Fig. 2 Temperature dependent heart rate/min of zoea I (A), zoea II (B) and megalopa larvae (C) of *Hyas araneus* reared at two different seawater P_{CO_2} (open circle: 420 µatm CO_2; closed circle: 3300 µatm CO_2; Mean±SE). Different letters indicate significant differences between temperatures within one treatment (lowercase letters: 420 µatm CO_2; uppercase letters: 3300 µatm CO_2).

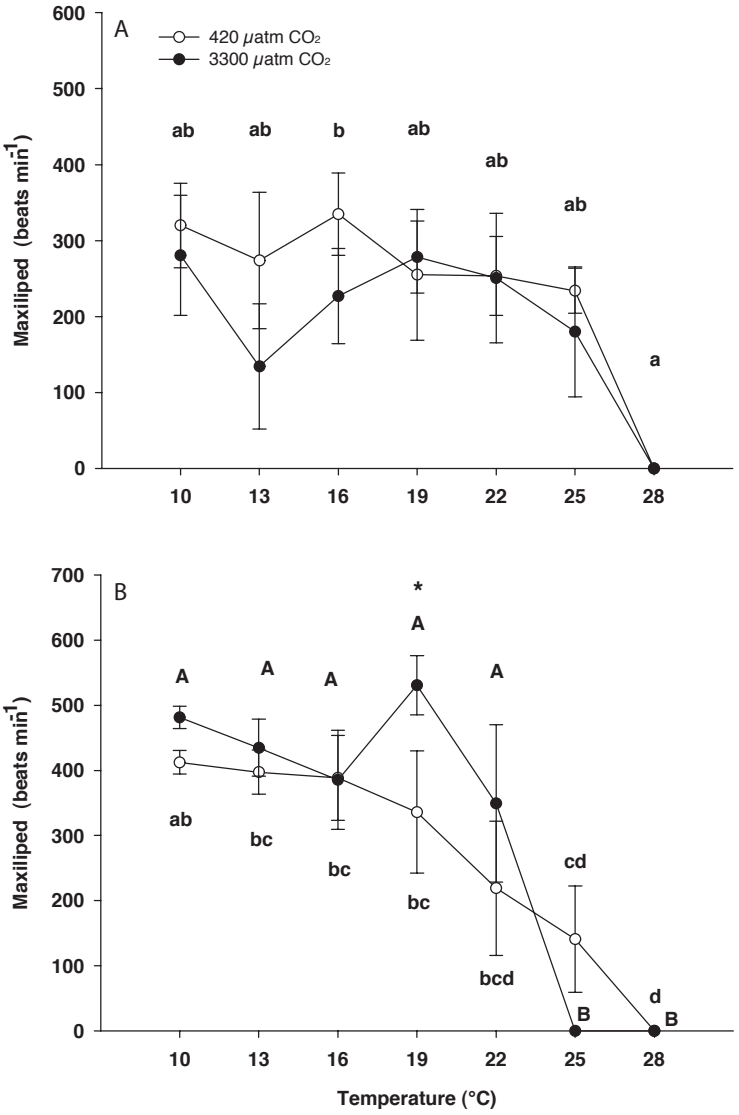


Fig. 3 Temperature dependent maxilliped beat rate of zoea I (A) and zoea II (B) of *Hyas araneus* reared at two different seawater P_{CO_2} (open circle: 420 µatm CO_2; closed circle: 3300 µatm CO_2. Mean±SE). Asterisks indicate significant differences between treatments at the same experimental temperature. Different letters indicate significant differences between temperatures within one treatment (lowercase letters: 420 µatm CO_2; uppercase letters: 3300 µatm CO_2).

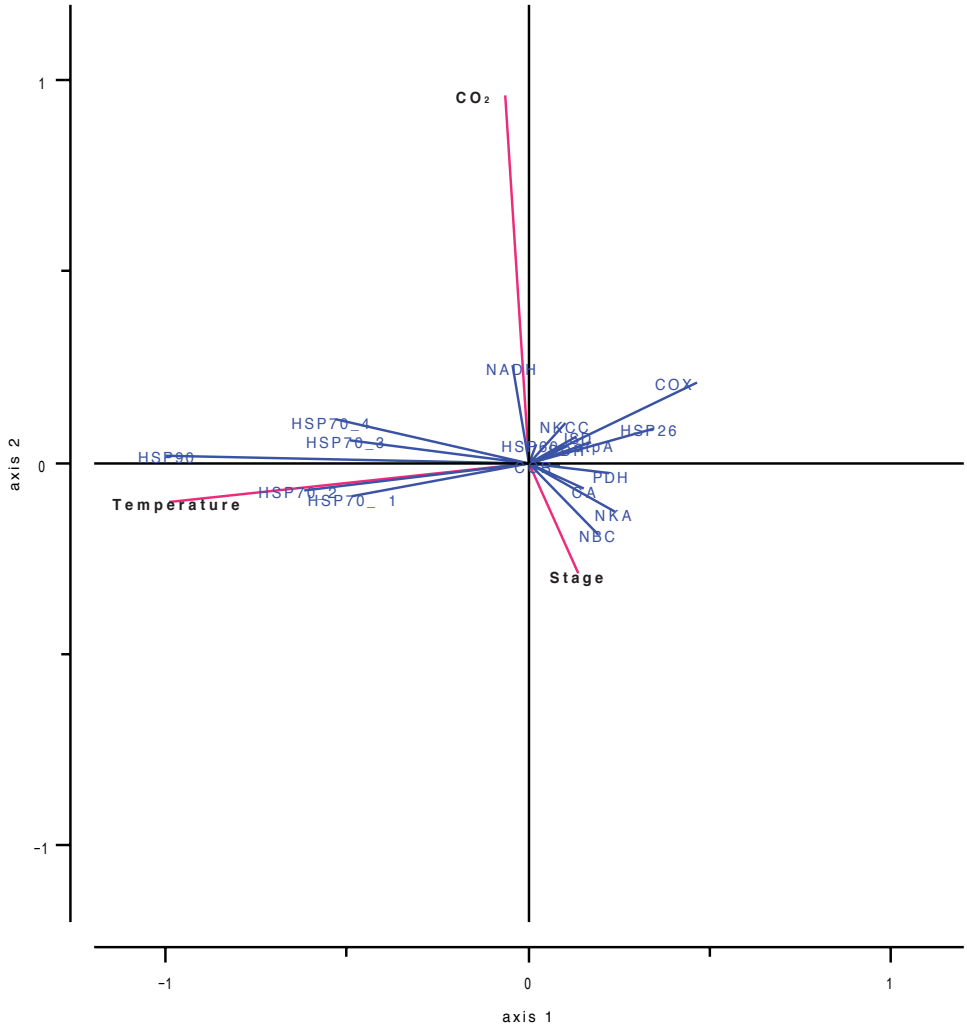


Fig. 4 The canonical correspondence analysis shows the relationship between gene expressions of the 18 investigated genes and the different factors CO_2, temperature and developmental stage. Length and alignment of lines illustrate the importance of the different factors on gene expression. See table 2 for specific abbreviations.

4 Discussion

4.1 Physiological responses of different life stages of *Hyas araneus* to elevated seawater P_{CO_2}

Physiological responses of marine organisms during hypercapnic exposure are closely linked to physiological adjustments due to changes in seawater carbon chemistry. Exchange of metabolic CO_2 between the extracellular fluid and the seawater is mainly achieved by means of diffusion. Increasing external (seawater) P_{CO_2}, at maintained diffusion gradients between the organism and seawater, cause haemolymph CO_2 to increase (Melzner et al. 2009b). The resulting changes in haemolymph pH are buffered to various extents via ion regulation to excrete CO_2 and thus maintain pH homeostasis (Melzner et al. 2009b, Whiteley 2011). As mentioned before, compensatory mechanisms are associated with energetic costs, while uncompensated extra-and/or intracellular acid-base disturbances can elicit metabolic depression and decrease oxygen affinity of the respiratory pigment (Whiteley and Taylor 1982, Langenbuch and Pörtner 2002).

The capacity of different life stages of the spider crab *Hyas araneus* to maintain intra- and extracellular pH homeostasis during hypercapnic exposure was determined in the present study in order to examine the ability of osmoconforming crustaceans to cope with future climate change (publication III, unpublished data). The results were related to studies on the effects of high CO_2 on metabolic rates and mitochondrial metabolic pathways to link the patterns of regulation with those seen in the animal's metabolism (publication III, unpublished data).

4.1.1 Acid-base status and regulation during hypercapnic exposure

CO_2 sensitivities of different marine taxa appear to be highly dependent on their capacities to regulate blood acid-base disturbances at elevated seawater P_{CO_2} via ion regulation (Melzner et al. 2009b). In decapod crustaceans, haemolymph acid-base regulation via ion transport molecules is mainly achieved through gill epithelia (Freire et al. 2008). Although our understanding of acid-base regulation and the contribution of different ion transporters is far from complete, it is thought that active ion transporters (e.g. H^+ATPase) and secondary active transporters (sodium proton exchanger (NHE); sodium bicarbonate co-transporter (NBC)) contribute to proton excretion while carbonic anhydrase (CA) and sodium potassium ATPase (NaK) play a role in maintenance of ion

gradients (Pörtner 2008, Melzner et al. 2009b). To investigate the molecular response and capacities of acid-base regulation in different life stages of *Hyas araneus*, the gene expression of different transporters and enzymes (NBC, NaK, CA, NHE) in larval stages kept at different levels of seawater PCO_2 was quantified (Fig. 4.1 and publication III). The data will be complemented by gene expression analyses in gills of adult males of *H. araneus* (Harms unpublished data).

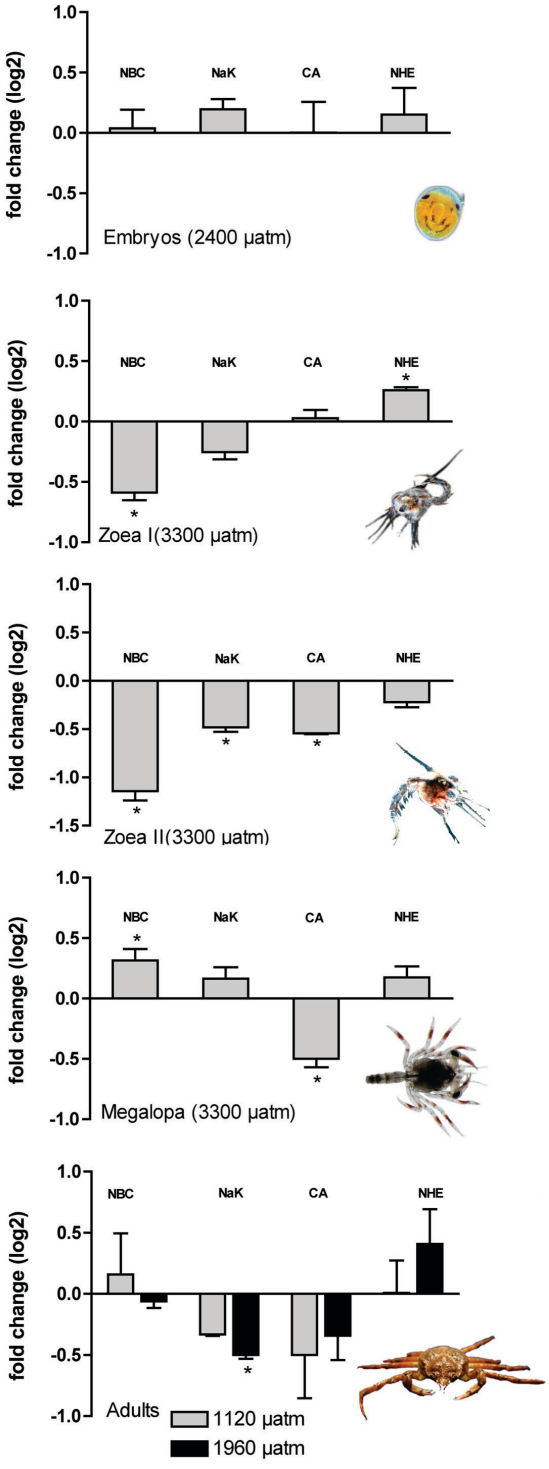


Fig. 4.1 Gene expression (calculated as log2 fold changes + SE in comparison to control (baseline)) of ion transporters and enzymes (sodium bicarbonate cotransporters NBC; sodium potassium ATPase NaK; carbonic anhydrase CA; sodium proton exchangers NHE) in gills of adult males (Harms unpublished data) and in eggs (unpublished data) and larvae of *Hyas araneus* (publication III) kept at elevated seawater PCO_2. Asterisks indicate significant differences between high CO_2 and control treatment.

In all stages, *H. araneus* showed no strong response in expression of these transporters and enzymes at high seawater CO_2 levels. If any, a down-regulation of the corresponding transporters could be seen in most life stages. In contrast, NaK and CA gene expression was 2–4 fold up regulated in strong osmoregulating crustaceans in response to salinity stress (Towle et al. 2011). Based on the molecular data, it can be assumed that all investigated life stages of *H. araneus* have weak abilities to regulate the extracellular pH (pH_e) actively during hypercapnic exposure via an increase in protein density of acid-base related enzymes and transporters.

It remained to be established whether the limited molecular response of ion transporters and enzymes is mirrored in low capacities of extracellular acid-base regulation in *H. araneus*. Measurements of extracellular pH in larvae of *H. araneus* are difficult to achieve due to the small size of the larvae. However, low capacities for ion regulation found in adults suggest the same pattern in larvae and embryos (Charmantier 1998).

In adult spider crabs *H. araneus* from an Arctic population of Svalbard (Norway) kept at two different temperatures (5°C as control and 10°C as elevated temperature) and different seawater PCO_2 for long-term (10 weeks), extracellular pH decreased with increasing seawater CO_2 concentration (Fig. 4.2). Haemolymph PCO_2 at 1000 µatm was twice that of the control animals with a further slight increase at higher seawater CO_2 concentrations (1600 – 3000 µatm). A hypercapnia-induced increase in the haemolymph bicarbonate concentration (HCO_3^-) could be found at 1000 µatm CO_2 in animals kept at 10°C. Comparable pH_e values between the 390 µatm and 1000 µatm CO_2 treatments indicate a compensated haemolymph acidosis through compensatory increase in HCO_3^- concentrations at 1000 µatm. The accumulation of 5 mmol/l HCO_3^- remained below that of other decapod crustaceans (Truchot 1984, Cameron and Iwama 1987, Pane and Barry 2007) exposed to higher CO_2 levels.

There was a different regulatory response of *H. araneus* after medium-term (12 days) (Zittier et al. 2012) and long-term exposure (10 weeks) to elevated seawater PCO_2 (Fig. 4.2). The reduction of haemolymph pH with increasing seawater PCO_2 was less pronounced after 12 days of exposure. In cold- and warm-acclimated animals, the extracellular pH decreased by 0.1 units and 0.3 units, respectively, at a seawater PCO_2 of 3000 µatm (Zittier et al. 2012). Haemolymph pH decreased by 0.4 units after ten weeks of exposure to 3000 µatm and 10°C (Fig. 4.2). Furthermore, there was a positive correlation between seawater PCO_2 and haemolymph PCO_2 after 12 days of exposure resulting in a 5-fold increase at 3000 µatm in warm-acclimated animals. After ten weeks, the haemolymph

P_{CO_2} did not differ in animals exposed to 1000 µatm and 3000 µatm. The most striking difference was seen in the HCO_3^- accumulation. The haemolymph HCO_3^- concentrations increased almost linear with the seawater CO_2 concentration after a medium-term exposure (Zittier et al. 2012), while after ten weeks the haemolymph HCO_3^- concentration increased at 1000 µatm followed by a significant decrease at 2000 and 3000 µatm, respectively (Fig. 4.2). Long-term exposure (10 weeks) might also limit the ability of adult *H. araneus* to actively compensate for haemolymph acid-base disturbances at medium seawater P_{CO_2} (1000 µatm) as the HCO_3^- accumulation remained below that of animals exposed for 12 days.

The data suggest a compensatory response of *H. araneus* via HCO_3^- accumulation positively correlated with the seawater P_{CO_2} concentration after a medium-term hypercapnic exposure. Despite the elevated haemolymph HCO_3^- concentration, haemolymph pH decreased significantly between control and the highest CO_2 treatment (Zittier et al. 2012, Fig. 4.2). Proton-equivalent ion exchange and the flexibility to respond to acid-base disturbances are reduced at low extracellular pH in the marine worm *Sipunculus nudus* (Pörtner et al. 2000b). The ion transporter NHE and NBC contributed to the acid-base regulation in *S. nudus*, but at a lower rate at reduced pH_e. The NHE and NBC can be found in the gills of crustaceans and are involved in acid-base regulation (Tresguerres et al. 2008). HCO_3^- produced via carbonic anhydrase hydration of CO_2 and an active uptake from the seawater can be released into the plasma by means of NBC. Lower NBC capacities at lower pH_e would thus limit an active accumulation of HCO_3^- and the compensation of acid-base disturbances. Reduced transporter capacities at low extracellular pH can be due to changes in transporter activities or densities of transporters. The gene expression and, thus, abundance of NHE and NBC was not affected by seawater P_{CO_2} in *H. araneus* adults (Fig. 4.1). Soleimani et al. (1995) suggested that NHE activity is predominantly regulated by pH with a constant number of transporters whereas another important transporter, the H^+-ATPase, is mainly regulated by HCO_3^- via changes in transporter abundances. Gene expression of H^+-ATPase was affected by elevated seawater CO_2 concentration in adults of *H. araneus* after a 10 week exposure (Harms unpublished data) revealing the same pattern as haemolymph HCO_3^- concentrations with a strong up-regulation at 1000 µatm followed by a decrease at 2000 µatm.

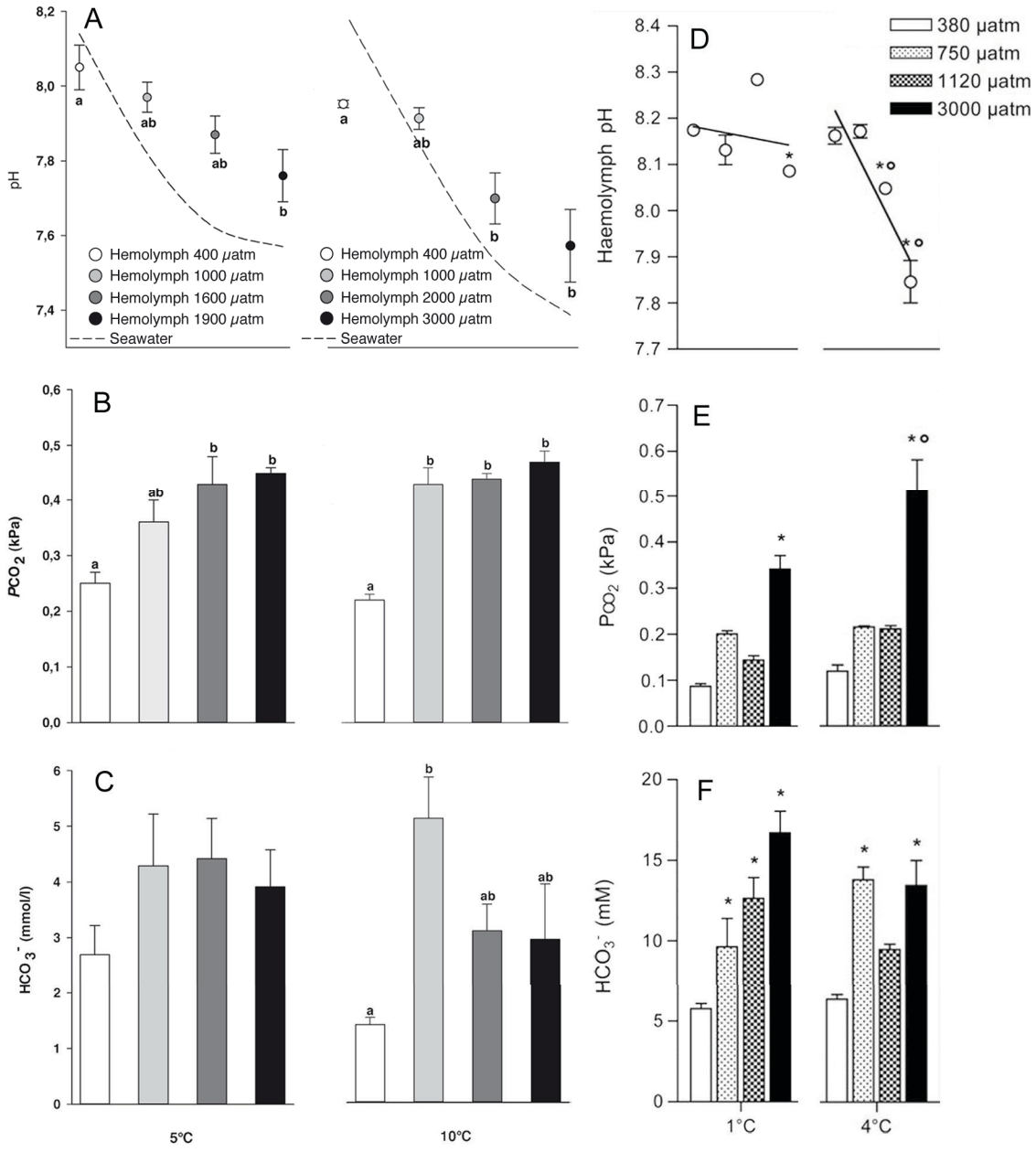


Fig. 4.2 **A-C** Haemolymph acid-base status of male Arctic spider crabs *Hyas araneus* after a 10 week exposure to different seawater PCO_2 levels and different temperatures. Different letters indicate significant differences between treatments. Mean ± SE. N = 4-8.

D-F Haemolymph acid-base status of cold (1°C) and warm (4°C) acclimated Arctic spider crabs *Hyas araneus* after a 12-day exposure to different seawater PCO_2 levels. Asterisk indicates significantly different from animals under control CO_2 levels at the same temperature. Degree significantly different between cold- and warm-acclimated crabs. N = 4–6 (Zittier et al. 2012)

A&D) pH_e, B&E) PCO_2 (kPa) and C&F) bicarbonate (mmol/l).

It seems that Arctic *H. araneus* has low capacities to fully compensate hypercapnia-induced acid-base disturbances via a sufficient increase in the haemolymph HCO_3^- concentration at high external P_{CO_2} (≈ 2000µatm). This leads to progressive decrease in the haemolymph pH entailing a limitation of ion transporter. Consequently, the haemolymph HCO_3^- concentration and buffer capacity decreases after a long-term hypercapnic exposure.

However, extracellular pH of *H. araneus* did not decrease to the same extent as the acidified seawater and was still 0.2 units higher than the pH of the surrounding seawater at 3000 µatm (Fig. 4.2). These findings suggest that it is at least partially compensated by the (albeit not significant) higher HCO_3^- concentration compared to control animals (Fig. 4.2).

One explanation for the low ability to regulate acid-base disturbances could be that pH_e regulation is energetically costly. Deigweiher et al. (2010) showed a hypercapnia-induced rise in the fractional cost of ion regulation in gills of Antarctic notothenioids. Adult *H. araneus* have a low metabolism, mirrored in low oxygen consumption rates (Fig. 4.4), compared to other decapod crabs (Thurberg et al 1973, Klein Breteler 1975), which might restrict the abilities to compensate for haemolymph acid-base disturbances.

Special emphasise in terms of acid-base regulation capacities should be given to embryos of some marine taxa. The embryos of e.g. fish, cephalopods and crustaceans inhabit an egg capsule during their development. Respiratory gases have to pass the egg wall, which represents a natural barrier for these gases. As respiration of *H. araneus* embryos depends on the developmental stage and increases with development (Petersen and Anger 1997, Fig. 4.10), respiratory CO_2 should accumulate within the egg capsule and would rise further at high seawater CO_2 concentrations.

As *H. araneus* embryos seem to have low capacities for active ion regulation, at least on a molecular basis (Fig. 4.1), exposure to high seawater CO_2 might elicit acid-base disturbances within the egg. However, there was no effect of elevated seawater CO_2 on egg pH, bicarbonate concentration and P_{CO_2} (Fig 4.3) when late stage III embryos of *H. araneus* were exposed to two different seawater P_{CO_2} of 450 µatm and 2400 µatm for 14 weeks. In both CO_2 treatments egg pH was low whilst P_{CO_2} and bicarbonate levels were extremely high compared to the extracellular compartment of adults. The measured parameters represent the egg fluid and extra- and intracellular fluid of embryos as a whole and make statements about the effect of a low egg fluid pH on the embryo acid-base status difficult. Unfortunately, there is almost no data available on the acid-base status of egg fluid and embryos in marine ectotherms. In eggs of the cuttlefish *Sepia officinalis*, P_{CO_2} of the perivitelline fluid (PVF) increased and pH decreased with development and embryonic

wet weight (Gutowska and Melzner 2009). However, the PVF pH of around 7.2 in *S. officinalis* was considerably higher than the mean egg pH of *H. araneus*, whereas PCO_2 at 0.25 kPa was lower than in *H. araneus* eggs and was negatively affected when embryos were exposed to high seawater CO_2 (Hu et al. 2011). Sedlacek (2008) reported even lower intracellular pH values of 5.4 for non-diapause eggs of copepod crustaceans. The methodical resolution might be a problem as the high HCO_3^- and PCO_2 levels as well as a relatively high standard deviation might limit the possibility to detect small changes due to high seawater CO_2 concentrations.

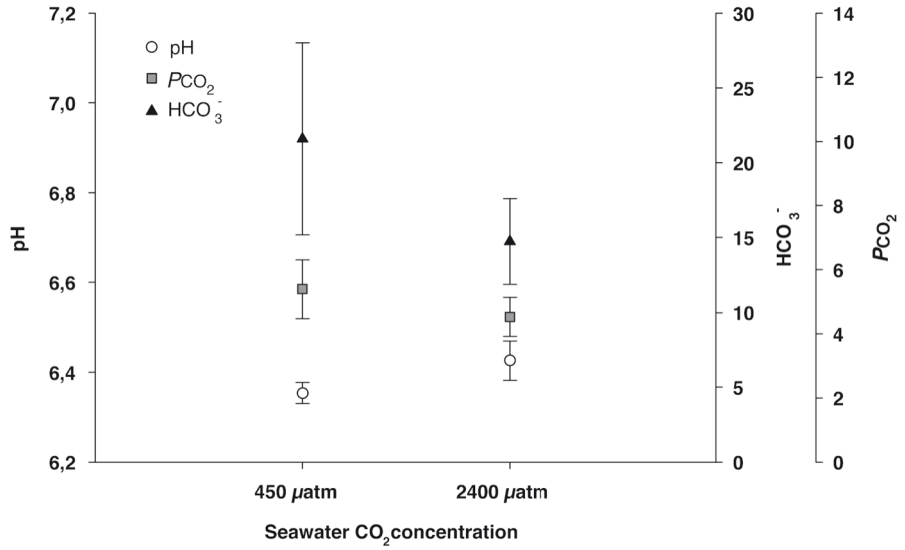


Fig. 4.3 Acid-base status of eggs of the Arctic spider crab *Hyas araneus* after 14 weeks of exposure to different seawater PCO_2 levels (450 µatm, 2400 µatm). egg pH (white circle), egg PCO_2 (kPa)(grey square) and egg bicarbonate (mmol/l) (black triangle). Mean ± SE. n = 9-10 egg batches

4.1.2 Effects of elevated seawater PCO_2 on metabolic rate and metabolic pathways in different life stages of *Hyas araneus*

There was no effect of elevated seawater PCO_2 or temperature on oxygen consumption in *Hyas araneus* adults from a temperate population (Sweden) exposed to different seawater PCO_2 (1000 – 2000 µatm) and different temperatures (10°C as control and 16°C as elevated temperature) (Fig. 4.4).

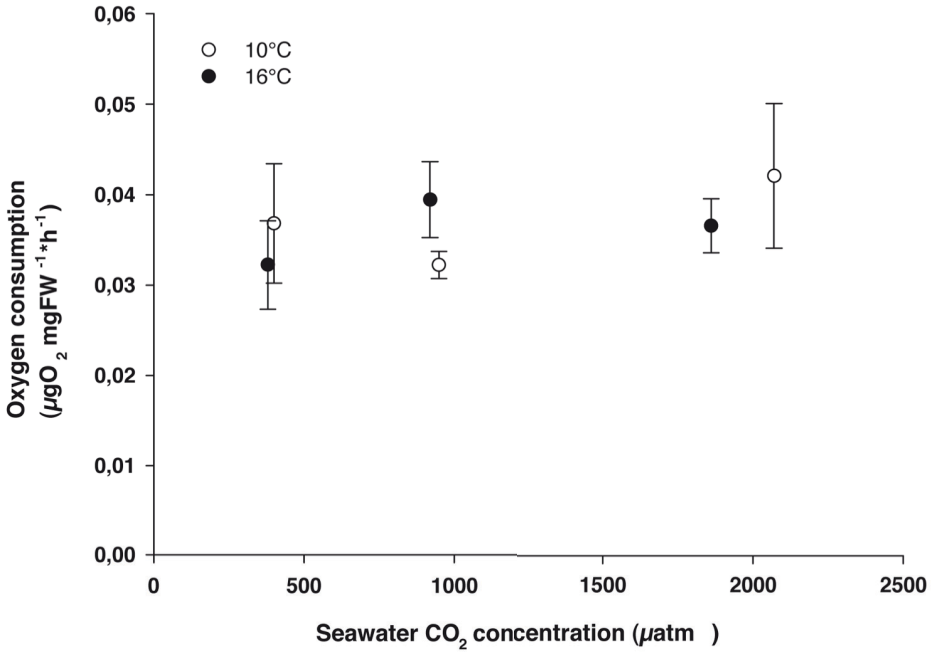


Fig. 4.4 Oxygen consumption of males of the temperate spider crab *Hyas araneus* adults after 10 weeks of exposure to different seawater P_{CO_2} levels and different temperatures (open circle 10°C, closed circle 16°C). Mean ± SE. n = 5-9

As discussed above, an active HCO_3^- accumulation and pH compensation by means of ATP-consuming ion transporters at a medium seawater CO_2 concentration (1000 µatm) might be accompanied by elevated energy demands, while an uncompensated pH_e at high seawater CO_2 (2000 µatm) may elicit a metabolic depression. However, ion regulation and metabolic depression proceed on a cellular level of different tissues and might not be reflected in whole-animal respiration. Furthermore, shifts in tissue and/or whole-animal energy budgets during hypercapnic exposure might occur. Overall gill energy turnover was constant under pH compensated hypercapnia, while energy demands for ion exchange, protein and RNA synthesis increased in Antarctic notothenioids suggesting a shift in the gill energy budget (Deighweiher et al. 2010). Besides ion regulation, calcification might be another energy-consuming process during exposure to high seawater CO_2 and a rise in calcification costs might compensate for metabolic depression of tissues.

Development in crustaceans is characterised by regular moulting events, including metamorphosis in some life stages. At regular intervals, crustaceans shed the old cuticle and a new cuticular matrix has to be synthesised and mineralised after moulting (Roer

and Dillaman 1984). During moult, calcium is reabsorbed from the old cuticle and partly deposited in storage structures (Luquet and Marin 2004). These ion movements and deposition during development and growth are energy-consuming processes, as they require active transport via a series of ATP consuming enzymes, such as Ca-ATPase (Roer and Dillaman 1984, Luquet and Marin 2004). Shell deposition and/or maintenance and elevated calcification rates, respectively, might explain elevated metabolic rates in marine calcifiers exposed to hypercapnic conditions (Wood et al. 2008, Stumpp et al. 2011a).

While calcification of *H. araneus* megalopa larvae was compromised at a seawater P_{CO_2} of 3000 µatm (Walther et al. 2011), electron microscope images did not show any changes in the carapace of adult *H. araneus* after exposure to high seawater CO_2 levels (Eva Klumpen personal observation, Fig. 4.5) indicating no carapace dissolution under conditions of elevated seawater P_{CO_2} in adults. There was no visible evidence of a detached and crenated cuticle at elevated seawater P_{CO_2} as it e.g. could be found in the edible crab *Cancer pagurus* (Eva Klumpen personal comment).

Carapaces of all animals used for experiments in the present thesis were covered with epibionts suggesting an intermoult stage. The potentially negative impact of a non-compensated haemolymph pH on calcification processes and the associated increase in energy costs may only become obvious during moulting, when ion movements and deposition are accomplished. Post-moult calcification under hypercapnic conditions took twice as long in the blue crab *Callinectes sapidus* (Cameron 1985). Furthermore, the $CaCO_3$ formation in crustaceans takes place in the shell fluid compartment at 0.3 to 0.5 pH units above that of the haemolymph (Cameron and Wood 1985). The observed decrease in haemolymph pH in adult *H. araneus* due to hypercapnic exposure will increase the pH gradient between the haemolymph and the shell fluid and more energy might be needed to maintain the shell fluid pH. This mismatch has the potential to adversely affect calcification and growth of this species.

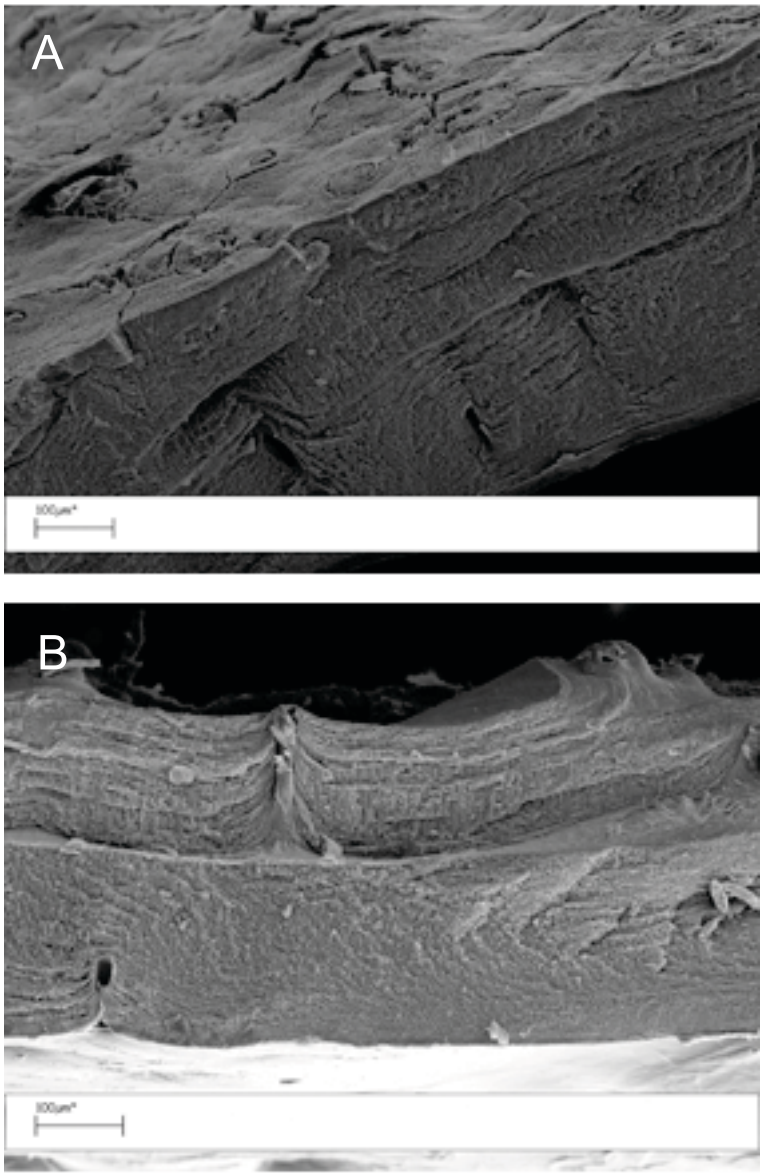


Fig. 4.5 Scanning electron micrographs of *Hyas araneus* cuticle after 10 weeks of exposure to different seawater P_{CO_2} levels. (picture by Eva Klumpen). A) Animals exposed to 400 µatm CO_2 B) Animals exposed to 2400 µatm CO_2. There was no visible evidence of a detached or crenated cuticle at elevated seawater CO_2.

A metabolic depression can be indicated by a repression of genes of the citric acid cycle and the electron transport system (van der Meer et al. 2005). In order to investigate whether high seawater CO_2 (> 3000 µatm) elicits a metabolic depression, transcript levels of several genes responsible for the mitochondrial energy metabolism were measured in different larval stages of *H. araneus* exposed to 420 µatm and 3300 µatm (publication III).

There was no indication for a hypercapnia-induced metabolic depression in the

gene expression of various genes involved in the citric acid cycle and the electron transport system in *H. araneus* larvae. The majority of genes representing mitochondrial metabolic pathways were up-regulated under hypercapnic conditions (Table 5 in publication III). An increase in whole-animal mRNA levels of these genes could indicate a compensation of elevated energy demands during exposure to elevated seawater P_{CO_2} as we assume that the strong increase in expression should result in increased protein levels. Elevated energetic costs should be accompanied by higher metabolic rates, which was not the case in *H. araneus* larvae (Fig. 1 in publication III). The results imply that standard metabolism under high seawater CO_2 can only be maintained due to higher numbers of enzymes from the citric acid cycle and the electron transport system. Elevated transcript levels of corresponding enzymes might be related to a depression in enzyme activities at elevated seawater CO_2 due an inhibition of rising intra- and extracellular bicarbonate concentrations (for details see discussion publication III). Elevated seawater as well as atmospheric CO_2 inhibited mitochondrial respiration and respiratory enzymes (Gonzàles-Meler et al. 1996, Drake et al. 1999, Strobel et al. 2012). Bicarbonate inhibited citrate synthase in mouse kidney mitochondria (Simpson, 1967) and activates adenylyl cyclase, which produces the second messenger cAMP, which is involved in enzyme regulation by phosphorylation and also transcription factor regulation (Acin-Perez 2009, Tresguerres et al. 2011). Thus, an increase in transcript levels of genes from the citric acid cycle and the electron transport system could be a prerequisite to maintain standard metabolic rates at high seawater CO_2.

An up-regulation of the majority of genes representing mitochondrial metabolic pathways was also evident in *H. araneus* adults after a ten week exposure to elevated seawater P_{CO_2} (Harms unpublished data) revealing the same pattern as haemolymph HCO_3^- concentrations with a strong up-regulation at 1000 µatm followed by a decrease at 2000 µatm.

4.2 CO_2 sensitivities of early life history stages and spawners: evaluation of potential bottlenecks

Different life cycle stages of benthic calcifiers are known to be differently affected by high seawater P_{CO_2} (Kurihara 2008). However, studies focusing on specific effects of high CO_2 on several life stages of one species are scarce. The present thesis examined the response of embryos, larvae, adults, and spawners of the spider crab *Hyas araneus* to elevated seawater P_{CO_2} from the systemic to the molecular level attempting to determine the stage-specific characteristics of CO_2 tolerance as it has been done for other stressors like temperature (Fig 4.7). Some physiological responses of different life stages of *H.*

araneus to elevated seawater P_{CO_2} have been discussed above (see chapter 4.1). This chapter will focus on the life stages, which have been proposed to be most sensitive to environmental stress: the early ontogenetic stages (embryos, early larvae) and the ovigerous females (Fig. 4.6).

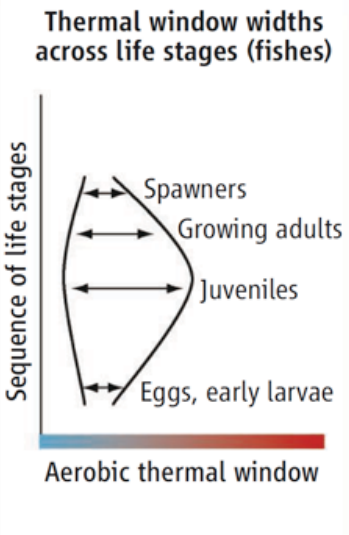


Fig. 4.6 The aerobic thermal window of different life stages of fish (from Pörtner and Farrell 2008). Position and width of windows can differ between life stages.

The early post-hatching larval stage of crustaceans (zoea I) has been identified as a relatively tolerant developmental stage also when exposed to extremely high seawater P_{CO_2}. Hypercapnic exposure did neither affect dry weight and elemental composition (growth) nor survival of the zoea I in different crustacean species (Arnold et al. 2009, Walther et al. 2010, 2011, Bechmann et al. 2011). The main response observed in post-hatching larval crustaceans exposed to elevated seawater P_{CO_2}, was slower development (Walther et al. 2010, Bechmann et al. 2011, Keppel et al. 2012). However, if elevated seawater CO_2 levels lead to longer developmental times in zoea larvae, studies of CO_2 effects on time-dependent functions would create a classic experimental design problem (Pörtner et al. 2010a). How to distinguish age-related effects from the effects of elevated CO_2 levels, when high CO_2 leads to different stages at a given time point? By sampling once per larval stage, previous studies suggested that there is no effect of elevated seawater P_{CO_2} on the growth of the first larval stages of crustaceans (Arnold et al. 2009, Walther et al. 2010, 2011). However, no differences in post-moult dry weight between larvae exposed to control and high CO_2, under which they needed longer time to moult into the subsequent stage, would indicate that at high seawater CO_2 a longer time period is needed to gain the same amount of weight as control larvae. This would imply reduced growth rates in early larval stages of crustaceans exposed to elevated seawater CO_2 levels. Thus,

dry weight should be measured at several time points in larval development to avoid misleading conclusions. Such measurements have not been conducted yet.

The underlying mechanisms responsible for the observed developmental delay are still unknown. Higher energetic demands for maintenance of intra- and extracellular homeostasis and calcification have been proposed for sea urchin larvae as possible mechanisms resulting in less energy available for somatic growth and a slower development (Stumpp et al. 2011a,b). So far, there are no data available concerning the energy budget of crustacean larvae during exposure to elevated seawater P_{CO_2} within and/or at a given time point in the moult cycle.

Ocean acidification studies disentangling the effects of larval age from those of high seawater CO_2 levels on physiological parameters have been carried out on sea urchin larvae with continuous development (Stumpp et al. 2011a, Martin et al. 2011), but not on species with discrete development (e.g. crustaceans). In crustaceans, the fitness of planktonic larvae is influenced by pre-hatching conditions, termed *carry-over effects* (Anger 2006). Carry-over effects were proven in *Chasmagnathus granulata* with an increase in the ability to hyper-osmoregulate in the first zoea stage after embryonic exposure to reduced salinities (Charmantier et al. 2002). The developmental delay in the early larval stages of crustaceans exposed to elevated seawater P_{CO_2} suggest that high seawater CO_2 levels constrain early larval development with unknown mechanistic background, which might become more obvious in the later larval stages by carry-over effects. However, in crustaceans, most studies dealt with effects on post-embryonic (post-hatching) larval stages but disregarded effects of seawater P_{CO_2} on embryos. This critical transition phase between embryos and early post-hatching larvae and any carry-over effect has not comprehensively been explored.

The development of physiological processes in the first larval stage of *H. araneus* in response to elevated seawater P_{CO_2} was determined in the present thesis in order to observe if changes in physiological responses occur at a certain time point and to examine the mechanisms underlying the observed developmental delay of the first larval stages in response to high CO_2 exposure (publication I). The results were linked to a comparative time series study indicating hypercapnia-induced carry-over effects between embryos and the first zoea stage (publication II). In order to determine if a CO_2 induced disruption of female-egg interactions could be responsible for the observed carry-over effects, brooding behaviour was monitored in ovigerous females of *H. araneus* (unpublished results).

4.2.1 Effect of pre-hatching seawater P_{CO_2} on the development of physiological processes during development of the first larval stage of *Hyas araneus*

Development of *Hyas araneus* zoea I larvae exposed to control seawater P_{CO_2} during pre-hatching phase was not delayed in response to acutely elevated seawater P_{CO_2} exposure during the larval phase (publication I). This is not in accordance with previous studies (Walther et al. 2010) and might be due to higher rearing temperatures used in my study. Development of crustacean larvae is characterized by a high degree of plasticity and depends on environmental conditions. Environmental temperature is one main driving force for developmental duration in crustacean larvae (Anger 1983). Temperature has a much stronger influence on developmental duration of *H. araneus* larvae at the cold end of the thermal tolerance window and the increased developmental duration due to a decrease in temperature is stronger than the acceleration caused by an equal increase in temperature (Anger et al. 1983, Walther et al. 2010). The negative impact of elevated seawater P_{CO_2} (3000 µtm) on larval developmental time in zoea larvae reared at 3°C vanished at higher rearing temperatures (9°C and 15°C) in the previous study on *H. araneus* (Walther et al. 2010). Thus, a rearing temperature of 6°C, as used in my study, might have been too high to detect a CO_2 induced developmental delay in *H. araneus* zoea I larvae (publication I). These findings indicate synergistic effects of temperature and seawater CO_2 concentration on mechanisms eliciting the developmental delay in crustacean larvae exposed to hypercapnic conditions. Both environmental factors seem to influence each other and effects might counteract each other.

One aim of my thesis was to detect the mechanisms responsible for the observed hypercapnia-induced developmental delay in the first larval stages of crustacean. Despite equal developmental times in *H. araneus* larvae reared at 490 and 2400 µatm (publication I), I was able, following the development of physiological processes, to show age-related differences in growth and energy demands in zoea I larvae reared at different seawater P_{CO_2} levels. As earlier studies sampled only once per larval stage (Arnold et al.2009, Walther et al. 2010, 2011), these differences might not have been detected and led to the assumption that the early post-hatching larval stage (zoea I) is a relatively tolerant developmental stage in terms of ocean acidification.

Under hypercapnic exposure, *H. araneus* zoea I larvae exhibited higher energy demands a few days before moulting into the second stage (zoea II). Oxygen consumption rate had reached a steady state level under control conditions, while there was a rise in oxygen consumption rate continuously leading to a maximum 1.5-fold increase beyond control level in larvae exposed to high seawater P_{CO_2} (Fig. 1 in publication I). As metabolic rates of larvae comprise the energy demand for both maintenance and swimming activity,

the hypercapnia induced elevation in the energetic costs was discussed and explained by increased activity shortly before moulting (see discussion publication II). The observed higher metabolic costs during pre-moult events at high CO_2 exposure likely deplete energy stores of zoea I larvae. Developmental duration and successful moulting of decapod crustacean larvae highly depend on energy reserves accumulated from food (Anger 1987). However, it is unlikely that higher metabolic costs during pre-moult events in larvae exposed to high seawater CO_2 concentrations elicit delayed moulting, as accumulated energy reserves should be sufficient to assure a successful moulting. The pre-moult phase is a comparatively short period within the moult cycle of crustacean larvae and elevated oxygen consumption rates in *H. araneus* zoea I larvae were only detected shortly before moulting into the second larval stage (day 36 post-hatching) (Fig 2 in publication I). Different critical points have been found in the interaction between energy accumulation from food and developmental duration in crustacean larvae (Anger 1987). One of them is termed ''point of reserve saturation (PRS)''. If larvae pass that point under conditions of sufficient food supply and reserve accumulation, they will successfully moult to the subsequent stage independent of food availability. The second critical point in the interaction between reserve accumulation from food and developmental duration in crustacean larvae is called ''point of no return (PNR)''. If unfed larvae had passed about 70% of the maximum possible survival time under starvation, they were not able to recover when re-fed and stayed in their present stage of development with no return to a complete moult cycle (Anger and Dawirs 1981).

The PRS is reached after one third to one half of total developmental duration of the first larval stage (Anger 1987). With a developmental duration of about 43 days, *H. araneus* zoea I larvae would have passed the PRS before the 1.5-fold increase of metabolic costs in larvae exposed to high CO_2 concentrations on day 36 post-hatching.

The effects of high CO_2 exposure on the metabolism did not influence zoea I survival (publication I). It seems that the zoea I stage of *H. araneus* is able to compensate for the elevated costs associated with the development in a high PCO_2 environment. This compensation might affect the performance of later stages by carry-over effects due to depletion of energy reserves (Dupont et al. 2012). The observed severe impact of hypercapnic exposure on later larval stages of crustaceans (Arnold et al. 2009, Walther et al. 2010, 2011) might therefore be explained by energy depletion through compensatory mechanisms in the early larval stages.

Energy depletion and energy accumulation and the associated growth and weight increment are highly relevant factors substantially shaping crustacean larval survival and developmental duration (Anger and Dawirs 1981, Anger 1987). The relevance of these

factors for larval survival and development under hypercapnic conditions becomes most obvious when comparing results of publication I and II.

Elevated seawater P_{CO_2} affected zoea I dry weight increment independent of pre-hatch history (high CO_2 during the pre- and post-hatching phase vs. normocapnic CO_2 during the pre-hatching phase and high CO_2 during the post-hatching phase). An exposure to high seawater P_{CO_2} elicits an initial time period with low dry weight increment followed by a developmental period of increasing dry weight until a certain time point in development when larvae gained the same amount of weight as control larvae (Fig. 4.7). However, zoea I larvae exposed to elevated seawater P_{CO_2} during the pre- and post-hatching phase had a longer period of low weight increment and needed longer to gain the same amount of weight as control larvae (Fig. 3 in publication II) compared to larvae acutely exposed to high seawater P_{CO_2} concentrations after hatching (Fig. 3 in publication I).

Beside the differences in dry weight increment in pre-exposed and acutely exposed larvae, we observed extremely divergent survival rates and developmental times. There was no effect of seawater CO_2 concentration on the survival rate and developmental duration in *H. araneus* zoea I larvae exposed to elevated CO_2 after hatching (publication I). Contrary, when embryos were pre-exposed to elevated P_{CO_2}, 64 % more of them died under continued CO_2 exposure during the zoea I phase and stage duration until moulting into zoea II was extended by 21 days at high CO_2 levels (publication II) (Fig. 4.8).

Divergent survival rates and developmental times of in pre-exposed and acutely exposed larvae might be due to differences in accumulation of energy reserves. I estimated the two critical points in the interaction between reserve accumulation from food and developmental duration in crustacean larvae (PRS and PNR) for *H. araneus* zoea I larvae with different pre-hatching history (publication I, publication II). The PRS should be reached between approximately day 15 and day 22 in larvae exposed to elevated seawater P_{CO_2} after hatching and between day 16 and day 24 in larvae exposed to high CO_2 during pre-and post-hatching phase. Maximum survival time during starvation of zoea I larvae reared under control seawater P_{CO_2} was 26 days at 3°C and 19 days at 9°C (publication II). Based on these findings, the PNR was calculated and interpolated for *H. araneus* zoea I larvae with different pre-hatching history. The PNR should be reached on day 16 in zoea larvae reared at elevated seawater P_{CO_2} after hatching and day 19 in pre-exposed zoea. When comparing differences in dry weight increment due to different pre-hatching history, these critical points should be considered (Fig. 4.7) as they relate to survival and moulting success. When *H. araneus* zoea I larvae acutely exposed to hypercapnic conditions after hatching reached the PNR and PRS, respectively, dry weight increased

continuously and larvae gained almost the same amount of weight as control larvae (Fig. 4.7 A). Zoea I larvae pre-exposed to high seawater P_{CO_2} concentrations before and after hatching passed the PNR long before the increasing dry weight equalled that of control larvae (Fig. 4.7 B).

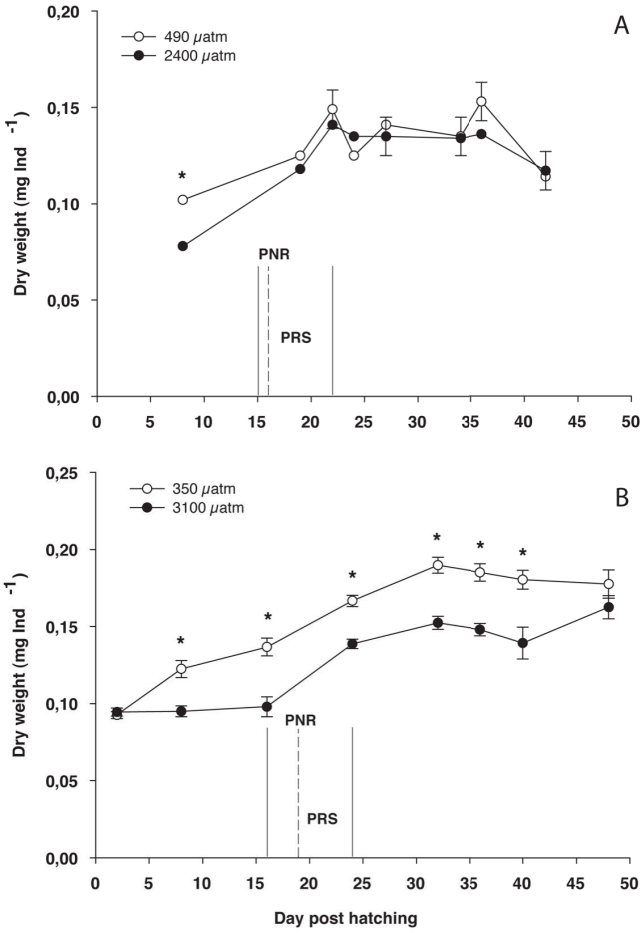


Fig. 4.7 **A** Growth pattern (dry weight) of zoea I larvae of *Hyas araneus* reared under two different seawater P_{CO_2} levels (490 µatm CO_2: white circles; 2400 µatm CO_2: black circles). Mean ± SE. Asterisks indicate significant differences between treatments on the same developmental day. N = 8 **B** Dry weight of zoea I larvae of *Hyas araneus* pre-exposed to elevated P_{CO_2} during the pre-hatching phase and reared under two different seawater P_{CO_2} levels (350 µatm CO_2: white circles; 3100 µatm CO_2: black circles) during time of development. Mean ± SE. Asterisks indicate significant differences between treatments on the same developmental day. PNR: ''point of no return'' (dashed line); area of PRS: ''point of reserve saturation'' (between solid lines) n = 8

As the PNR and PRS are reached during the first third of larval development, it might be a critical phase within the larval development determining the recruitment success. *H. araneus* larvae depend on immediate food supply for a successful development. Earlier studies showed that a sufficient food supply determines developmental duration and survival of crustacean larvae (Anger and Dawirs 1981, Dawirs 1983). Elevated seawater P_{CO_2} caused a reduction in zoea I feeding rates at the beginning of the moult cycle, when embryos were pre-exposed to elevated seawater P_{CO_2} (publication II). The higher mortality and prolonged development found in *H. araneus* zoea I larvae pre-exposed to high seawater CO_2 during the pre-hatch phase might be attributed to reduced feeding rates and slower dry weight increment before reaching the PNR. This assumption is further supported by the fact that survival of pre-exposed zoea I started to decreased considerably when larvae passed the PNR (Fig. 4.8).

The lower survival of pre-exposed zoea I larvae could also be due to the higher seawater P_{CO_2} of 3100 µatm used for experiments. However, exposure to even higher seawater CO_2 levels of 3300 µatm did not considerably increase zoea I mortality of *H. araneus* (publication III).

The insufficient growth and dry weight increment during the beginning of the moult cycle in *H. araneus* zoea I larvae pre-exposed to elevated seawater P_{CO_2} before hatching might be attributed to either reduced feeding or elevated energy demands. Elevated respiration rates at increased seawater P_{CO_2} in zoea I larvae two days post-hatching (Fig. 2A in publication II) could only be observed in pre-exposed, but not in acutely exposed zoea I larvae, indicating that high seawater CO_2 levels might also affect the hatching process. The underlying physiological mechanisms still need to be explored. However, during this transitional phase of metamorphosis, larvae may be especially sensitive to environmental stressors.

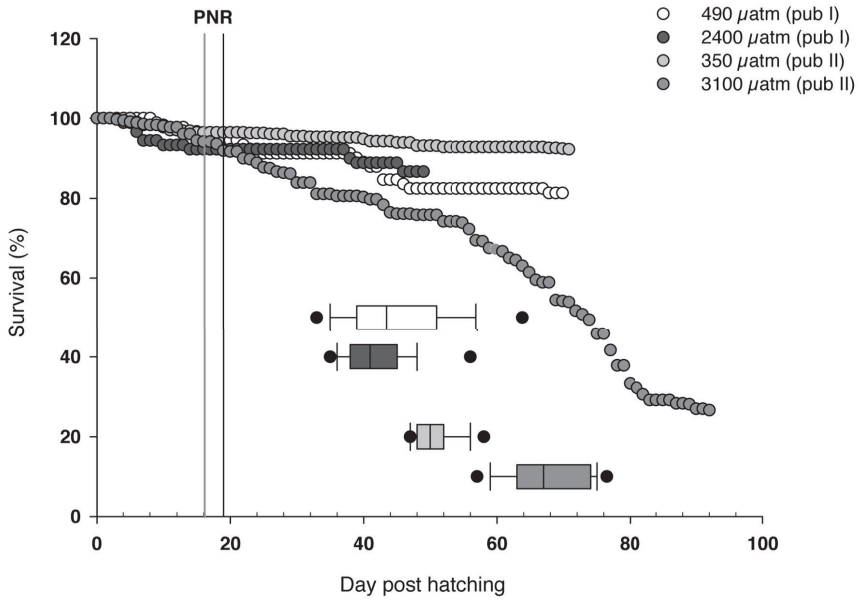


Fig. 4.8 Survival (%) of zoea I larvae of *Hyas araneus* reared under 490 µatm (white circles) and 2400 µatm (dark grey circles) (6°C), respectively, and zoea I kept at 350 µatm CO_2 (light grey circles) and 3100 µatm CO_2 (grey circles) (4°C) after the two weeks of the respective pre-exposure of eggs. Data were collected from hatching onward until larvae were either dead or moulted to the second stage. Box whisker plots show developmental time of zoea I larvae of *H. araneus* from hatching until moulting to the second stage (same colour code for the different treatments). Box limits represent 25th and 75th percentiles, the line within the box marks the median and whiskers indicate 90th and 10th percentiles. PNR: ''point of no return'' for zoea I larvae acutely exposed to high seawater PCO_2 concentrations after hatching (grey line) and pre-exposed during the pre-hatching phase (black line).

Elevated respiration rates two days post hatching indicate an uncompensated energetic cost associated with maintenance at high seawater PCO_2. Respiration comprised 16 %, while growth comprised 11 % and excretion 71 % of the total energy assimilated by food intake and 2 % was lost as cast exoskeletons in zoea I larvae of *Carcinus maenas* (Dawirs 1983). Assuming a similar energy budget for *H. araneus* zoea I larvae, any increase in the cost of maintenance two days post hatching would reduce the amount of energy available for other processes like growth (Fig. 4.9). Metabolic rates of larvae comprise the energy demand for both maintenance (standard metabolic rates SMR) and swimming activity (active metabolic rates AMR). Storch et al. (2011) suggested that

swimming costs were between 17 % and 22 % of total metabolic rate in larval stages of the Chilean kelp crab *Taliepus dentatus*. It is possible that a shift in energy budget occurs in pre-exposed zoea I larvae of *H. araneus* with more energy spent on maintenance and less for swimming. As a dilemma, lower swimming activity causing lower feeding rates reduces energy availability and may thereby exacerbate the reduction in growth (Fig. 4.9).

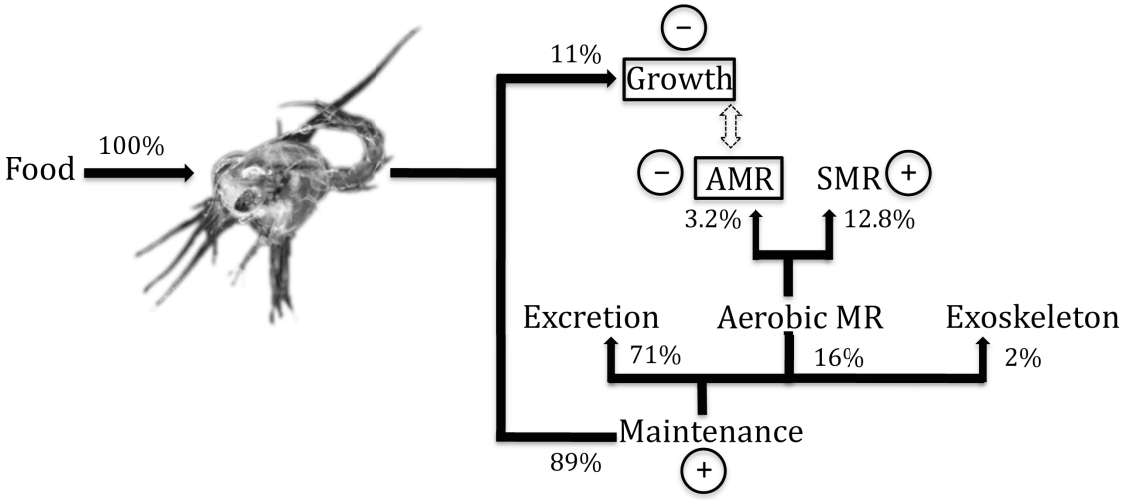


Fig. 4.9 Diagram of the energy budget for zoea I larvae of *Hyas araneus*. Percentage of different processes was calculated after Dawirs (1983) and Storch et al. (2011). CO_2 induced shifts in the energy budget are indicated by + and –. + depicts a possible CO_2 induced increase in the corresponding process. – depicts a possible CO_2 induced decrease in the corresponding process. The dashed arrow indicates the relationship between active metabolic rate (AMR) and larval growth. Metabolic rate (MR), standard metabolic rate (SMR).

Not only during the transitional phase of remodelling between embryo and larvae, early life stages may be especially sensitive to environmental stressors, but also during the embryonic phase. As the fitness of planktonic larvae is influenced by pre-hatching conditions, effects of high seawater CO_2 on embryos might carry over to the first larval stage resulting in low zoea I survival. Another experiment has been performed to investigate effects of elevated seawater P_{CO_2} on embryos and ovigerous females. Non-ovigerous and ovigerous females carrying late stage III embryos of *H. araneus* were exposed to two different seawater P_{CO_2} of 450 µatm and 2400 µatm for 14 weeks. The effect of high CO_2 exposure on the embryonic acid-base status has been discussed in the previous chapter (4.1). The high mortality rates of pre-exposed zoea I larvae cannot be

explained by an altered acid-base status within the egg capsule as egg pH, P_{CO_2} and HCO_3^- were not affected by the seawater CO_2 concentration (Fig. 4.3). However, the methodical resolution might be a problem and might limit the possibility to detect small changes. Lower survival rates of pre-exposed zoea I larvae might be due to modified behaviour of the female. The effect of elevated seawater CO_2 concentrations on the supply of oxygen to the embryos through specific female brooding behaviour is discussed in the next section.

4.2.2 Brooding behaviour of females during hypercapnic exposure

Like in many marine decapod crustaceans, *Hyas araneus* embryos are protected by the female, and eggs are attached to the female's pleopods. To guarantee oxygen availability in theses compact egg masses, specific female behaviours could be demonstrated in some species (Fernández et al. 2000, Baeza and Fernández 2002). Parental care involves costs related to the provision of oxygen to the embryo. Fernández et al. (2000) showed that oxygen consumption of brooding females could be almost twice as high as standard metabolism in non-brooding spider crabs *Maja squinado*.

There is already evidence that exposure to elevated seawater P_{CO_2} leads to a shift in energy budgets eliciting reduced somatic and reproductive growth in echinoderms (publication II, Stumpp et al. 2012). In brooding females of crustaceans, a CO_2 induced shift in the energy budget might constrain sufficient provision of oxygen to the embryo as less energy can be invested in parental care. This might have crucial consequences for embryos and the survival of subsequent larval stages. Embryonic development is oxygen limited (Fernández et al. 2003). Oxygen limitation would be exacerbated by enhanced embryonic oxygen demand or a limiting role for female ventilation. Progressive oxygen limitation goes hand in hand with CO_2 accumulation from respiration and thereby exacerbates exposure to elevated P_{CO_2}.

Embryonic oxygen consumption increased with time in control embryos, with no significant effect of hypercapnia (2400 µatm). However, energy demands of embryos were (albeit not significant) higher after two weeks of exposure to high seawater CO_2. Hypercapnic exposure led to an enhanced ventilation activity in female *H. araneus* despite the same embryonic oxygen consumption after 14 weeks (Fig. 4.10). On average, each female flapped its abdomen twice per hour at control seawater P_{CO_2}, while high CO_2 exposure increased the number to 11 times per hour. As abdominal flapping is associated with an increase in oxygen availability in the centre of the brood mass (Baeza and Fernández 2002), the observed high mortalities in zoea I larvae exposed to elevated seawater during pre- and post-hatching phase might not be due to a constricted provision

of oxygen to the embryo by the female. Unfortunately, no data on the flapping frequency of females were obtained after two and eight weeks of exposure to high seawater CO_2 and an initially limited provision of oxygen to the embryo cannot be excluded.

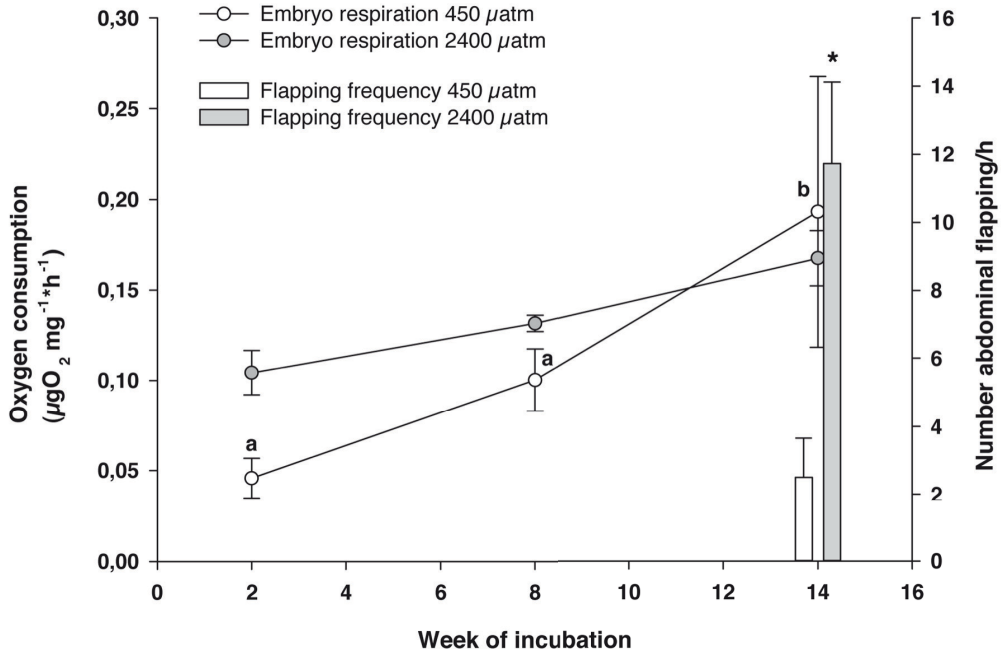


Fig. 4.10 Oxygen consumption of embryos of *Hyas araneus* exposed to two different seawater P_{CO_2} (450 µatm CO_2 white circle, 2400 µatm CO_2 grey circle) and number of abdominal flapping per hour of ovigerous females measured after 14 weeks (450 µatm CO_2 white bar, 2400 µatm CO_2 grey bar). Mean ± SE. Eggs: Different lowercase letters indicate significant differences between weeks of incubation within one treatment. N= 4-6. Females: Asterisks indicate significant differences between treatments. N=6

Our understanding of the underlying mechanisms linking active brood care and oxygen provision in crustaceans is far from being complete. Abdominal flapping of female crabs was observed to increase the oxygen availability in the center of the egg masses (Bazea and Fernández 2002). Chela and pereiopods were used to pierce the embryo masses and appendages were directed to the female mouthparts after eggs were pierced (Bazea and Fernández 2002). A high number of mechano- and chemosensory setae are located at the dactylus of the pereiopods and in the mouth region of crustaceans (Ache 1982, Schmidt and Gnatzy 1984). Abdominal flapping triggered by release of pheromones through eggs during time of hatching have been reported in ovigerous crabs (de Vries et al.

1991). The observed changes in female brooding behaviour at high seawater CO_2 might be due to a chemo-responsive behaviour, as it has been shown for the chemo-responsive ''sniffing response'' in an intertidal crustacean (de la Haye et al. 2012).

An altered female brooding behaviour at elevated seawater P_{CO_2} in *H. araneus* should have consequences for the female's energy budget with females putting more effort in oxygenation of broods. Acid-base status of ovigerous and non-ovigerous females were compared to investigate if ovigerous females spend less energy on acid-base regulation due to energy depletion through elevated costs of brooding. There were no differences in extracellular and intracellular (muscle) acid-base status of ovigerous and non-ovigerous females of *H. araneus* (data not shown). This indicates that elevated costs of brooding under high CO_2 are not at the expense of acid-base regulation. Further studies are needed to evaluate possible higher energy demands or/and shifts in the energy budget of ovigerous females in a high CO_2 world.

4.3 Synergy between CO_2 and temperature: implications for future spatial distribution of *Hyas araneus*

Future scenarios predict a concomitant increase of seawater CO_2 concentrations and temperature with both factors in synergy challenging marine species and ecosystems. Interaction between these environmental factors on the physiology of marine organisms can result from physiological mechanisms affected by both factors (Pörtner et al. 2005). Different underlying physiological mechanisms might be affected. 1) Ion regulation: Interactions of elevated seawater P_{CO_2} and temperature elicit strong acid-base disturbances leading to reduced protein functional capacity via lowered pH_e and thereby lower temperature tolerance. 2) Metabolism: An uncompensated drop in extracellular pH can have a depressing effect on aerobic energy metabolism, which will likely reduce the capacities to withstand temperature extremes. 3) Elevated seawater P_{CO_2} affects the heat shock response (higher or lower heat shock protein abundance). 4) Function of respiratory pigment: A lower haemolymph pH limits the functionality of the respiratory pigment and, therefore, a sufficient oxygen supply at high temperatures.

To test whether these biological processes set temperature tolerance during hypercapnic exposure, changes in transcript levels of several genes responsible for ion regulation, metabolism and heat shock response were determined in different larval stages of *Hyas araneus* exposed to elevated seawater P_{CO_2} before and after larvae were heat shocked. On the whole animal level, oxygen consumption, heart rate and activity during acute warming was measured to determine thermal windows of different larval

stages exposed to elevated seawater P_{CO_2} in order to examine their temperature tolerance and a possible limitation due to high CO_2 exposure (publication III).

The low capacity of *H. araneus* to compensate for hypercapnia induced extracellular acid-base disturbances did not elicit a metabolic depression and the expression of genes of mitochondrial metabolic pathways was mainly affected by elevated seawater CO_2 with no synergistic effects of high temperature and high CO_2 (publication III). The results have been discussed in section 4.1. In the next section I will focus on the gene expression patterns of cellular heat stress response in *H. araneus* larvae exposed to elevated seawater P_{CO_2} in order to align molecular responses to whole organism performance and to reveal mechanisms affected by the synergistic action of high CO_2 and high temperature. Furthermore, the role of the respiratory pigment of the crustaceans, haemocyanin, and its oxygen binding characteristics under hypercapnia will be discussed for adult *H. araneus* (unpublished data) as systemic oxygen supply strongly affects thermal tolerance windows (Frederich and Pörtner 2000) and respiratory protein function may be of central importance sustaining tolerance to hypercapnia and elevated temperatures.

As different populations along a latitudinal gradient are adapted to different thermal regimes and might possess different thermal tolerance windows, they might be affected to a different degree by increasing ocean temperatures. Acute temperature tolerance windows of larval stages from different populations of *H. araneus* will be compared to those of larvae of another spider crab species (*Pugettia producta)* (publication III, unpublished date) in order to assess the vulnerability of populations and species to ocean temperature changes and to discuss implications for their future spatial distribution considering the aspect of narrower thermal windows at high seawater CO_2.

4.3.1 Temperature tolerance of *Hyas araneus* exposed to elevated seawater P_{CO_2}

Larval thermal windows of different larval stages of *Hyas araneus* were determined in the present thesis (publication III) and will be compared to the adult thermal window examined in a previous study (Walther et al. 2009). However, different populations of *H. araneus* were sampled. Adults were collected from the Helgoland population of *H. araneus*, while larvae were obtained from the Gullmarsfjord (west coast of Sweden). The temperature range experienced by the Helgoland population of *H. araneus* is comparable to that of the population in the Gullmarsfjord with an annual mean temperature range of 2-17°C (www.weather.loven.gu.se/en/data.shtml). I could show that the three different larval stages of *H. araneus* display different upper thermal tolerance limits indicated by maximal oxygen consumption and heart rates detected at 25°C in zoea I

and zoea II in comparison to 22°C in megalopa larvae (publication III). Different temperature ranges in three larval stages have also been reported for the kelp crab *Taliepus dentatus* with the narrowest window found in the megalopa (Storch et al. 2011). It has been proposed for marine fish that early larval stages possess narrower thermal windows and, thus, are more stenothermal than adults (Pörtner et al. 2006). This assumption also holds true for *H. araneus* with an upper critical temperature of over 25°C in adults, 25°C in zoea stages and 22°C in the megalopa stage. The high sensitivity of the megalopa to environmental stressors has been found in previous studies suggesting that this larval stage is a physiologically sensitive bottleneck within the life cycle of decapod crustaceans (Walther et al. 2010, Storch et al. 2011).

Our knowledge of mechanistic principles in stenothermal polar fish might help to explain why thermal windows vary between different life history stages (Pörtner et al. 2006). Stenothermal fish have low standard metabolic rates and high Q_{10} values of baseline mitochondrial oxygen demand, which might reflect high activation enthalpies and therefore reduced maintenance costs (Pörtner et al. 2006). Reduced standard metabolic rates favour growth rates as a trade-off and at the cost of enhanced stenothermy. This pattern is applicable to the megalopa larvae of *H. araneus*. The lower respiration rates of *H. araneus* megalopa larvae at the rearing temperature of 10°C (publication III, Fig. 1) and higher growth rates compared to the more agile zoea stages (Anger et al. 1983) might narrow its thermal tolerance.

Different thermal tolerance windows of life history stages might also be due to morphological and physiological constraints and different capacities of central organs like the heart (Pörtner et al. 2006). Heart rates of adult and larval fish are temperature sensitive and increase with increasing temperature. Fish larvae displayed higher Q_{10} values of standard metabolic rates and heart rates than adult fish, although larval Q_{10} values for heart rate were lower than the values for standard metabolic rates (Pörtner et al. 2006). Pörtner et al. (2006) suggested that mechanisms responsible for temperature compensation in adult hearts are less or not developed in larval fish.

For *H. araneus* adults, Walther et al. (2009) reported a Q_{10} for heart rate of 1 between 10°C and 25°C. Q_{10} values were 1.5 (zoea I), 1.9 (zoea II) and 1.4 (megalopa) between 10°C and the critical temperature of 25°C in the zoea stages and 22°C in megalopa stage (publication III) and, thus, slightly higher compared to the adults. The cardiac activity of larval *H. araneus* seems to be more temperature sensitive triggered by mechanisms comparable to fish larvae and less developed mechanisms of temperature compensation might narrow the larval thermal window of *H. araneus* larvae.

In early larval stages of fish, developing cardiac performance and activity are not

yet fully linked to metabolic requirements as in adult fish (Pelster 2002). The concept of oxygen and capacity limited thermal tolerance (Pörtner 2001, Pörtner and Farrell 2008) is based on the assumption that a mismatch between oxygen demand and oxygen supply due to limited capacity of ventilatory and circulatory systems at temperature extremes limits the aerobic scope and, as a consequence, thermal tolerance (Frederich and Pörtner 2000). If metabolic requirements are insufficiently linked to cardiac activity, a lower Q_{10} for cardiac output than for oxygen consumption in early larval stages would lead to an early oxygen deficit during warming. Q_{10} values for heart rate and oxygen consumption were close to 1.5 between 10°C and 25°C in zoea stages of *H. araneus* (publication III). In *H. araneus* megalopa larvae, the Q_{10} value for oxygen consumption between 10°C and 22°C revealed a strong temperature dependency and was about 5, while the Q_{10} value for heart rate was much lower (1.4) (publication III). It seems that the lower metabolic rates of the megalopa stage led to enhanced stenothermy accompanied by a much stronger increase in oxygen demands with warming than observed in zoea stages. Elevated oxygen demands could not be covered by a sufficient increase in oxygen supply due to limited capacity of the circulatory system in the megalopa. The high thermal sensitivity of megalopa might be a trade-off for reduced energy expenditure making this larval stage a physiologically sensitive bottleneck within the life cycle of *H. araneus*.

Exposure to elevated seawater CO_2 narrowed the thermal window of all larval stages and the adults of *H. araneus* (Walther et al. 2009, publication III). The upper critical temperature shifted from above 25°C at control P_{CO_2} to 23,5°C and 21,1°C, when adults were exposed to elevated seawater CO_2 concentrations of 710 µatm and 3000 µatm, respectively (Walther et al. 2009). The shift of upper thermal limits in hypercapnia-exposed adults was attributed to stronger thermal stimulation of heart rate possibly as a response to lower oxygen tensions. As haemolymph oxygen levels set thermal tolerance windows in marine crustacean adults (Frederich and Pörtner 2000), function of the respiratory protein, haemocyanin, is of central importance during exposure to elevated seawater P_{CO_2} and elevated temperatures. Haemocyanin occurs freely dissolved in the haemolymph of crustaceans and its oxygen binding characteristics are of greatest importance for oxygen loading and CO_2 unloading of blood at the gills, oxygen transport and oxygen release and CO_2 loading in the tissue. The binding of oxygen to haemocyanin is (amongst others) affected by temperature, pH and CO_2 in crustaceans (Weber and Hagerman 1981, Jokumsen et al. 1981). Metabolic CO_2 is released at tissues and forms bicarbonate and protons. High proton concentrations lead in turn to a low haemolymph pH. High CO_2 concentration and low haemolymph pH cause more oxygen to be released from the protein (Schmidt-Nielsen 1975). *Vice versa*, low CO_2 concentrations and a more

alkaline pH favours the oxygen uptake at the gills. This effect is known as Bohr effect. However, CO_2 can lower the oxygen binding capacities even at a constant pH (Weber and Hagerman 1981) possibly by binding to the respiratory protein.

In a preliminary approach, the oxygen binding characteristics of haemocyanin of adult *H. araneus* were examined in a pH saturation diagram (unpublished data, Fig. 4.11). Animals were exposed to 390 µatm (control P_{CO_2}) and 1960 µatm (high P_{CO_2}) for ten weeks. Haemocyanin oxygen saturation was determined at different haemolymph pH and P_{O_2} to investigate the effect of elevated seawater P_{CO_2} on the relationship between pH_e, P_{O_2} and oxygen binding. Measurements started at different pH_e as hypercapnia-exposed animals had a lower *in vivo* pH of 7.7 compared to a pH of 8.0 in control animals. There was almost no decrease of oxygen saturation with pH at an oxygen partial pressure of 21 kPa, while the steepest decrease in oxygen saturation with pH_e was found at 1 kPa, 2 kPa and 5 kPa in animals from both treatments (Fig. 4.11). The steepness of the oxygen saturation isobars is an indicator for the pH sensitivity of haemocyanin as it describes the relationship between the changes in pH and the liberation of oxygen from haemocyanin. There was strong effect of seawater CO_2 concentration on the steepness of the 1, 2 and 5 kPa isobar. Haemocyanin saturation decreased from 100 % to 50 % between a pH_e of 8.1 and 6.8 at a P_{O_2} of 2/5 kPa and 7.4 at a P_{O_2} of 1 kPa, respectively, in control animals. At high seawater CO_2, haemocyanin became half-saturated between a pH_e of 7.5 and 7.3 and 7.1 at a P_{O_2} of 2/5 kPa and 1 kPa, respectively, suggesting an influence of seawater CO_2 the on haemocyanin molecule.

In crustaceans, the contribution of haemocyanin to the *in vivo* oxygen transport increases with decreasing haemolymph P_{O_2} (Lallier and Truchot 1989). Hypoxic exposure (4 kPa) of the shore crab, *Carcinus maenas*, elicited a strong decrease of haemolymph P_{O_2} from 14 kPa to around 1.5 kPa, which was accompanied by an increase in haemolymph haemocyanin levels and enhanced haemocyanin oxygen affinity (Lallier and Truchot 1989). Under normoxic conditions oxygen transport relied mainly on physically dissolved oxygen (80 %), while during hypoxia the major part of the oxygen was transported as oxyhaemocyanin (94-98 %). In *H. araneus*, the maximum steepness of the 1 and 2 kPa oxygen saturation isobars indicates high haemocyanin cooperativity implying that relatively small changes in pH are sufficient to deliver a large fractions of haemocyanin bound oxygen into the blood. Higher haemocyanin cooperativity might be a crucial strategy to cope with a lower *in vivo* pH_e at elevated seawater CO_2. At a lower pH_e, the pH range between the gills, at which oxygen loading of blood takes place, and metabolising tissue, at which oxygen is released, would be narrowed. The high haemocyanin cooperativity would favour oxygen unloading over a small pH range.

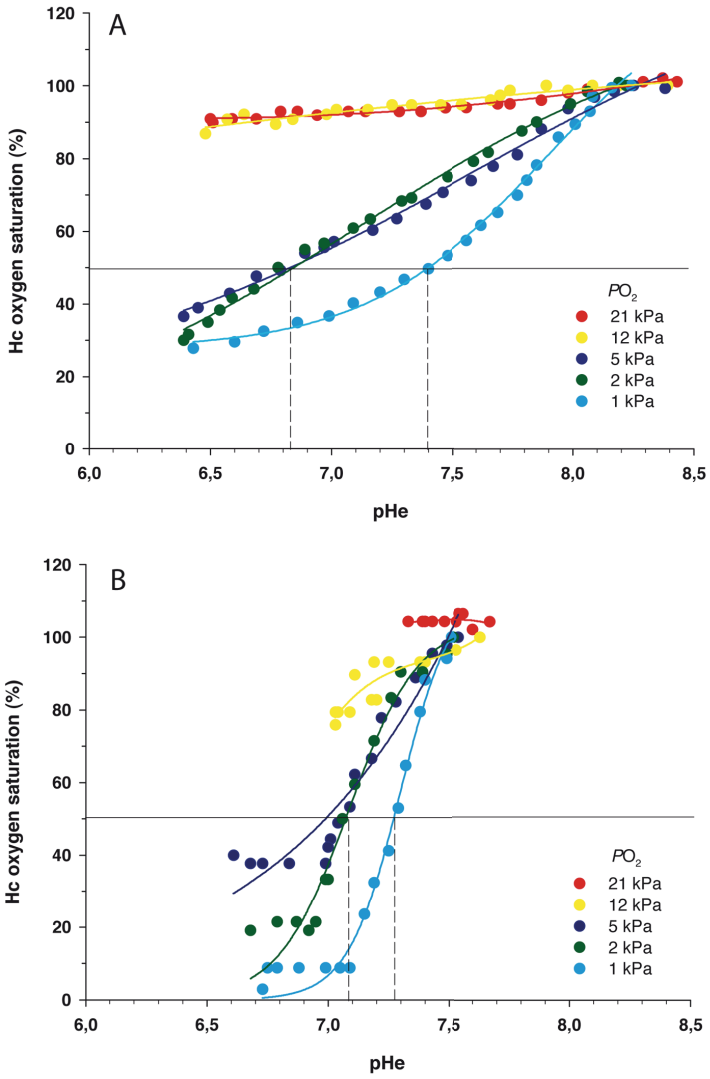


Fig. 4.11 Relationship between pH_e, haemocyanin oxygen saturation and PO_2 in adults of *Hyas araneus* exposed to control seawater PCO_2 of 390 µatm (A) and elevated seawater PCO_2 of 1960 µatm (B). The solid line indicates the half-saturation of haemocyanin with oxygen. Dashed lines indicate the corresponding pH_e for the half-saturation of haemocyanin.

These preliminary results (n = 1, haemolymph of one animal per CO_2 concentration) provide the first evidence that a hypercapnic exposure chances the pH sensitivity of the respiratory pigment at a low haemolymph PO_2 in crustaceans. This effect might become involved during acute warming. Haemolymph PO_2 decreased with increasing temperature in *H. araneus* adults down to values of around 2 kPa at critical temperatures of 21°C and 23°C in hypercapnia-exposed animals (Walther et al. 2009). At

elevated seawater P_{CO_2}, a lower haemolymph pH and a higher pH sensitivity of the respiratory protein at low P_{O_2} might act in concert narrowing the thermal tolerance of *H. araneus* through an oxygen limitation at lower temperatures. Further studies are necessary to substantiate the proposed mechanisms.

The earlier oxygen- and capacity limitation of thermal tolerance (OCLTT) of larvae and adults of *H. araneus* at elevated seawater P_{CO_2} was also reflected in the heat shock response (heat shock protein abundance). In *H. araneus* adults from the Gullmarsfjord (Sweden), the proteomic response to elevated seawater P_{CO_2} levels and permanent heat stress resulted in a higher abundance of heat shock protein (HSP) 70 (Harms, unpublished data). Zoea larvae of *H. araneus* showed a similar response at the gene expression level (publication III). Zoea larvae reared at elevated CO_2 of 3300 µatm showed higher expression of HSP70 and HSP90 than control larvae, when they were exposed to heat shock 10°C above the ambient temperature. A left shift of the three key characteristics (*T*on, *T*peak and *T*off) of the heat-shock response has been discussed in publication III. In marine organisms, OCLTT thresholds seem to be reflected in the threshold temperatures for the HSP response. *T*on was found to be close to the upper pejus temperature at which mortality starts to rise (Anestis et al. 2007), while *T*off was close to the upper critical temperature at which survival was strongly compromised (Tomanek and Somero 1999). As heat shock response and threshold temperature for HSP induction show a high degree of phenotypic plasticity (Buckley et al. 2001, Hamdoun et al. 2003) and are subject to acclimation, the results indicate a capacity to adjust passive thermal tolerance under CO_2 in larvae and adults of *H. araneus* (Fig. 4.12). Proteins might be less stable during hypercapnic exposure and thermal denaturation of the proteins might have to be prevented at lower temperatures leading to a left shift of *T*on and equal shifts in *T*peak and *T*off. However, a left shift of the heat shock response is a trade-off between maintenance of thermal tolerance and costs of thermal denaturation of the protein pool at lower temperatures.

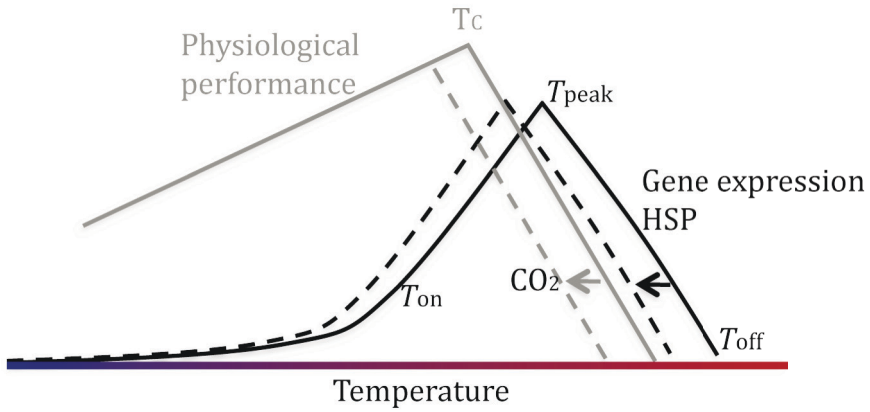


Fig. 4.12 Conceptual model of how hypercapnia-exposure shapes the physiological performance and concomitantly the heat-shock response (heat shock protein gene expression) of *H. araneus* during acute warming. High P_{CO_2} shifts the three key characteristics of the heat-shock response: Temperatures at which enhanced synthesis of HSPs first occurred (*T*on), temperatures of maximal induction of HSP synthesis (*T*peak) and temperatures at which HSP synthesis was heat-inactivated (*T*off) (adopted from Tomanek and Somero 1999). The synergistic effect of elevated seawater P_{CO_2} and heat shock cause a left shift of the heat shock response equivalent to the left-shift of the critical temperature (T_c) at which the physiological performance decreases.

A different physiological and heat shock response was found in the megalopa stage of *H. araneus*. There was no effect of seawater CO_2 concentration on the thermal sensitivity of heart beat rates and only slight differences in oxygen consumption during acute warming in *H. araneus* megalopa (publication III, Fig. 1&2). Furthermore, gene expression of HSP after a heat-shock of 20°C was not affected by seawater CO_2 level in the megalopa larvae (Table 5 in publication III). The data suggest that the high thermal sensitivity of the megalopa under control conditions is accompanied by the limited capacity of stress response mechanisms. Unlike the zoea stages, the megalopa stage seems to have low capacities to shift thermal limits or enhance the capacity for passive thermal tolerance, emphasizing the inflexibility or bottleneck characteristics of this larval stage (Fig. 4.13). This larval instar has already been suggested to be physiologically sensitive bottleneck within the life cycle of *H. araneus* in terms of ocean acidification and warming (Walther et al. 2010, 2011).

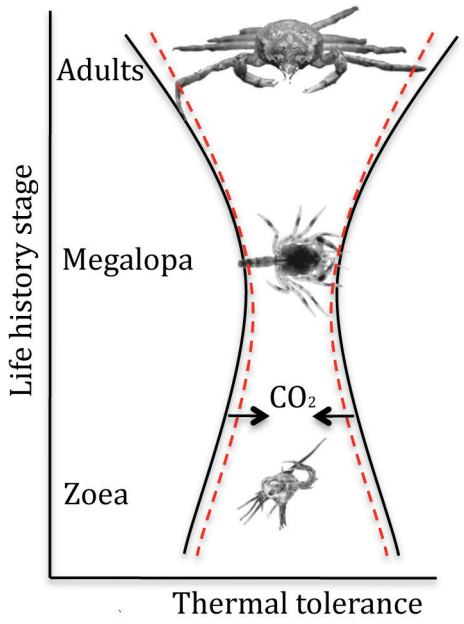


Fig. 4.13 Conceptual model of ontogenetic changes in the thermal tolerance of *Hyas araneus*. High seawater CO_2 concentration mainly affects the thermal tolerance of adults and zoea stages, while the low thermal tolerance of megalopa larvae might not be further limited at high CO_2.

4.3.2 Temperature tolerance of different population and species of spider crabs

The energetic demands during acute warming of spider crab larvae from different populations and of different species have been examined in the present thesis (publication III, unpublished data). The thermal tolerance of larvae of *Hyas araneus* was investigated in two populations: An Arctic from the Kongsfjord (Svalbard, 79°N) and a warm-temperate from the Gullmarsfjord (Sweden, 58°N). In the Kongsfjord the temperature ranges from 0-6°C (Svendsen et al. 2002), while larvae from the Gullmarsfjord experience ambient temperature between 2°C and 17°C (www.weather.loven.gu.se/en/data.shtml). *Pugettia producta* is a spider crab species, which is comparable to *H. araneus* in terms of its distribution range and life history stages. Just like *H. araneus, P. producta* shows a wide distribution range. While *H. araneus* is distributed from Arctic waters around Svalbard to boreal waters of the southern North Sea, *P. producta* can be found from boreal Chichagof Island, Alaska to subtropical Asuncion Point, Mexico. In contrast to *H. araneus*, it is able to exist and reproduce in warmer, subtropical waters.

Oxygen consumption rates of zoea larvae from the Arctic population of *H. araneus* doubled between 3°C and 9°C (Fig. 4.14). Oxygen consumption rates were constant between 9°C and 21°C in zoea I larvae, while there was a further increase between 9°C and 15°C in zoea II larvae followed by a significant drop at 21°C. Oxygen consumption rates of zoea I larvae were significantly higher than that of zoea II larvae at 21°C (Fig. 4.14). This pattern was not reflected in the temperate population of *H. araneus*. Oxygen consumption of both zoea larvae increased between the rearing temperature of 10°C and 25°C, followed

by a significant drop at the highest experimental temperature of 28°C (Fig. 4.14). These results indicate that the first zoea stage of Arctic *H. araneus* has a higher relative (not absolute) upper critical temperature than both temperate zoea stages. Furthermore, differences in thermal windows were identified in Arctic *H. araneus* zoea larvae, which was not the case in the temperate population. Arctic zoea II larvae displayed a narrower thermal window than zoea I larvae.

Larvae from the two different populations of *H. araneus* differ in their absolute thermal tolerance. Critical temperature limits, indicated by maximal oxygen consumption, were reached at 25°C in both temperate zoea stages of *H. araneus* (Fig. 4.14). Arctic *H. araneus* zoea II displayed maximal oxygen consumption at 18°C. The lower critical temperature limits were accompanied by a stronger thermal sensitivity of whole-animal respiration in both Arctic zoea stages. Arctic larvae had considerably higher Q_{10} value for oxygen consumption, especially at the low end of their temperature tolerance, compared to temperate larvae (Tab. 4.1). In Arctic larvae, the exceptional high Q_{10} (12.7) at 3°C above the rearing temperature is due to the Q_{10} method of extrapolation. However, the same method was used for all stages and populations, still resulting in a lower Q_{10} for temperate larvae. The results indicate permanent cold adaption in the Arctic population of *H. araneus*. Stenothermy and high sensitivity to warming might result from high Q_{10} values of oxygen consumption due to high thermal sensitivity of the proton leak and an associated dissipative heat loss during warming in cold-adapted, polar ectotherms (Pörtner et al. 2000b).

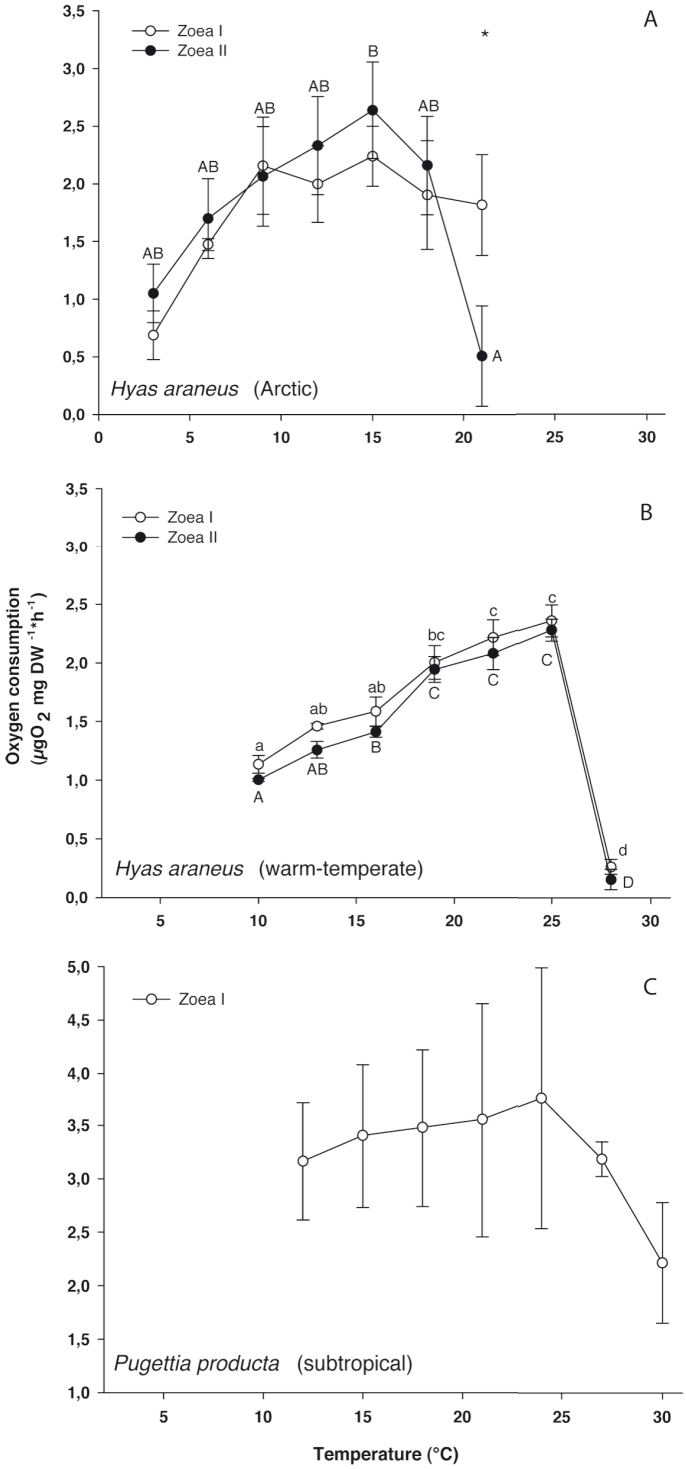


Fig. 4.14 Temperature dependent oxygen consumption (μgO_2 $mgDW^{-1}$ h^{-1}) of Arctic (A) and temperate (B) zoea I and zoea II of *Hyas araneus* and subtropical zoea I of *Pugettia producta* (C) (Mean ± SE). Asterisks indicate significant differences between life stages. Different letters indicate significant differences between temperatures within one life stage (lowercase letters: zoea I; uppercase letters: zoea II).

As high seawater CO_2 levels narrows the thermal tolerance window of *H. araneus* adults and larvae from temperate populations (Walther et al. 2009, publication III), a concomitant increase in ocean CO_2 concentrations and temperature might cause migration and a northern shift in the distribution limits of *H. araneus*. An increase in temperature in the North Sea during the last 40 years has already led to a dramatic decrease in the abundance of *H. araneus* around Helgoland (Walther et al. 2010). However, a migration and a northern shift in the distribution could lead to an increased degree of cold adaptation, which comes with enhanced stenothermy as a trade-off presumably entailing an increase of sensitivity towards ocean warming.

Table 4.1 Q_{10} for oxygen consumption in Arctic and temperate zoea I and zoea II larvae of *Hyas araneus* and zoea I larvae of subtropical *Pugettia producta*. Temperature is given as difference between rearing (control) and experimental elevated temperature.

Zoea I	**Increase in temperature (°C above rearing temperature)**					
Population/Species	**3°C**	**6°C**	**9°C**	**12°C**	**15°C**	**18°C**
Arctic *H. araneus*	**12.7**	**6.7**	**3.3**	**2.7**	**2.0**	**1.7**
Temperate *H. araneus*	**2.3**	**1.7**	**1.9**	**1.7**	**1.6**	**0.4**
Subtropical *P. producta*	**1.3**	**1.2**	**1.1**	**1.2**	**1.0**	**0.8**

Zoea II						
Population	**3°C**	**6°C**	**9°C**	**12°C**	**15°C**	**18°C**
Arctic *H. araneus*	**5.0**	**3.1**	**2.4**	**2.2**	**1.6**	**0.7**
Temperate *H. araneus*	**2.1**	**1.8**	**2.1**	**1.8**	**1.7**	**0.4**

The thermal tolerance window of *P. producta* zoea I larvae from a subtropical population (California, 37°N) was determined (Fig. 4.14). Unfortunately, no data for the second zoea stage could be obtained due to difficulties in larval culturing. Whole-animal respiration was almost thermally insensitive with constant Q_{10} values of around 1 (Tab. 4.1). There was a slight, but not significant decrease in oxygen consumption at 18°C above the rearing temperature. However, it was less pronounced than in zoea stages of Arctic and temperate *H. araneus*. Critical temperature limits might be encountered above a seawater temperature of 30°C. Larvae of the sampled subtropical population of *P. producta* experience ambient temperatures between 7°C and 17°C. Although larvae are exposed to a smaller temperature range during development compared to other *P. producta* populations further south (e.g. Mexico, 16-26°C), they still show a high temperature tolerance. At the corresponding rearing temperature, *P. producta* larvae displayed oxygen consumption rates, which were three times higher than those of Arctic and temperate *H. araneus* larvae. Adaptation to a thermal environment requires a balance between mitochondrial density, corresponding standard metabolic rates and as a result,

thermal upper and lower critical limits (Pörtner et al. 2000b). The investigated population of *P. producta* seems to be warm-eurythermal with high upper critical temperature limits. Larvae from the temperate *H. araneus* population showed an intermediate and Arctic larvae the narrowest thermal window suggesting a cold eurythermy for the temperate and a permanent cold stenothermy for the Arctic population. Although temperature tolerance of *P. producta* zoea I larvae was narrowed by elevated seawater P_{CO_2} (data not shown), larvae of warm-eurythermal species/populations might be less affected by a concomitant increase of seawater CO_2 concentrations and temperature due to wider thermal tolerance windows. This could lead to a higher reproductive success in areas of coexistence and shifts in species abundance, which could have strong ecological implications.

5 Conclusion

In order to draw a conclusive picture, the research objectives formulated in the introduction will be apprehended with respect to the experimental results:

1) Can different life cycle stages of the osmoconforming Hyas araneus regulate hypercapnia-induced acid-base disturbances? Does a disruption or regulation of the acid-base status compromises the metabolism of Hyas araneus?

Adults of osmoconforming *Hyas araneus* cannot fully compensate for hypercapnia-induced acid-base disturbances of extracellular compartments via bicarbonate accumulation or trans-epithelial ion transport. Extracellular pH decreased with increasing seawater CO_2 concentration, but was compensated for to various degrees by an increase in bicarbonate. mRNA expression of several genes important for acid-base regulation were either not affected by high seawater PCO_2 or mainly down-regulated in embryos, larvae and adults. There was no evidence for metabolic depression or compromised metabolism due to insufficient acid-base regulation. However, a shift in energy budget cannot be excluded. High seawater CO_2 levels had an influence on mitochondrial energy metabolism indicated by an up regulation of genes of the electron transport chain and citric acid cycle.

Depending on pre-hatch history and larval age, exposure to elevated seawater PCO_2 caused a rise in standard metabolic rates of *H. araneus* larvae. Elevated metabolic rates were paralleled by reduced growth rates in larvae pre-exposed to elevated PCO_2 during maternal care, possibly due to shifts in the larval energy budget.

2) *Are early developmental stages (embryos, larvae) the most CO_2 sensitive life cycle stages of Hyas araneus?*

Predicting CO_2 sensitivities of different life history stages of a species is difficult as different physiological mechanisms might be affected at different life stages. Adults and larvae (zoea) had efficient compensatory mechanisms to maintain homeostasis during hypercapnic exposure. Despite extracellular acid-base disturbances in adult *H. araneus*, exposure to elevated seawater CO_2 did not affect oxygen consumption, mortality or carapace structure. Effects of elevated seawater PCO_2 on oxygen consumption, weight and elemental composition in developing zoea I larvae of *H. araneus* were small, developmental duration and survival remained unaffected. These findings are in line with

previous studies on crustacean larvae, where elevated seawater P_{CO_2} did not influence development, mortality or growth in the first two zoea stages of the European lobster (Arnold et al. 2009) or zoea mortality and growth in *H. araneus* (Walther et al. 2010,2011).

Crustacean zoea larvae show relatively high survival rates under high levels of seawater P_{CO_2} in comparison to other marine invertebrate larvae (Dupont et al. 2008, Talmage and Gobler 2010). To maintain homeostasis, compensatory mechanisms might involve an up-regulation of enzymes of the mitochondrial energy metabolism, especially those involved in the electron transport chain. A hypercapnia-induced increase in transcript levels of these enzymes could be found in zoea larvae (publication III) and also in adult *H. araneus* (Harms unpublished data).

The outcome of the experiments underlines the hypothesis that early ontogenetic stages (embryos) and spawners are sensitive life stages of *H. araneus* (Fig. 5.1). Hypercapnic exposure leads to altered female brooding behaviour. This might have consequences for the female's energy budget with females increasing their effort in brood oxygenation. It cannot be excluded that embryos were affected by elevated seawater P_{CO_2} through less oxygen supply by the females. However, the transition from eggs to hatching larvae may be even more critical than progression within an individual stage per se and thus, depicts a putative bottleneck in the larval development of *H. araneus*. Zoea I larvae pre-exposed to high seawater P_{CO_2} during maternal care showed extremely high mortality rates and severe developmental delays due to potential hypercapnia-induced changes in the early larval energy budget leading to insufficient growth increment.

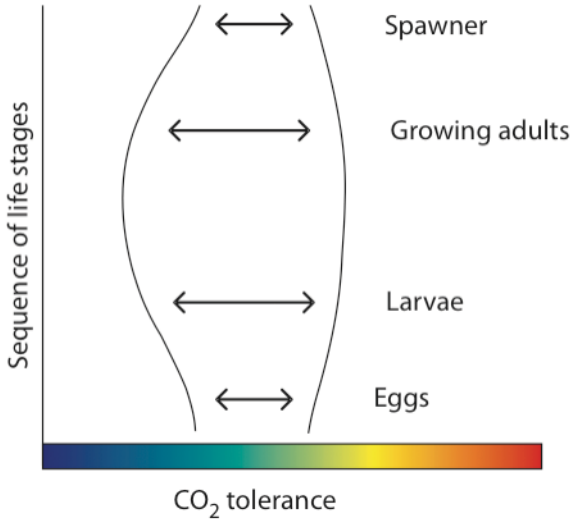


Fig. 5.1 Schematic model of ontogenetic changes of CO_2 tolerance in the spider crab *Hyas araneus*.

3) *Is the thermal tolerance of Hyas araneus larvae limited under elevated seawater P_{CO_2} due to synergistic effects of hypercapnia and temperature on metabolism, acid-base regulation and/or heat shock response?*

At elevated seawater CO_2 concentrations, upper thermal tolerance limits were lowered by around 3°C in zoea larvae of *H. araneus*. This suggests that high CO_2 and elevated temperatures act synergistically on physiological mechanisms of the larvae. The genetic approach of the present thesis supports the hypothesis that a shifted physiological temperature tolerance is reflected in changes in the heat shock response, namely the gene expression of heat shock proteins (HSP). Chronic exposure of larvae to elevated CO_2 increased the transcriptomic response to acute heat shock, while the mRNA expression of genes from other cellular processes (metabolism and ion regulation) did not respond to a synergistic treatment of high seawater CO_2 and acute heat shock.

Regarding the megalopa stage, neither the physiological measurements, nor the outcome of the genetic approach indicated a hypercapnia-induced narrowing of the thermal tolerance range. However, the stenothermy of the megalopa stage of *H. araneus* might reduce the possibility to detect differences in its thermal tolerance and also might prevent a further reduction of the thermal tolerance capacity due to hypercapnia-exposure.

The present thesis set out to pursue the question if the environmental variability during daily life can be correlated with capacities for CO_2 tolerance in marine crustaceans. It was postulated that osmoconforming, slow-moving inactive species commonly found in the deep sea and high latitudes are the species most at risk in terms of ocean acidification and warming (Pane and Barry 2007, Whiteley 2011). Summing up the present results, osmoconforming sublitoral crustacean, such as *H. araneus*, species are more tolerant towards ocean acidification than they were expected to be. It seems that *H. araneus* adults tolerate extracellular disturbances, raising the question whether this is an adaptive strategy or due to limited physiological capacities. Whiteley (2011) suggested that the low oxygen carrying and buffering capacity contributes to the low compensatory capacities in inactive species with low metabolic rates. However, a low, mainly passive, buffering of extracellular acid-base disturbances is characterized by several advantages and disadvantages. Maintenance of intra- and extracellular acid-base balance is accomplished to ensure continuity of normal physiological and metabolic processes (Henry and Wheatly 1992). At the extracellular level this involves maintaining the structural and functional integrity of the respiratory pigment haemocyanin. As the contribution of haemocyanin to

the *in vivo* oxygen transport increases with decreasing haemolymph P_{O_2} in crustaceans (Lallier and Truchot 1989) often displayed at elevated temperatures, uncompensated acid-base disturbances might affect oxygen transport at temperature extremes. This is further supported by the observed higher pH sensitivity of the respiratory protein at low P_{O_2} in hypercapnia-exposed adults of *H. araneus* and the previously reported lower heat tolerance at high seawater P_{CO_2} (Walther et al. 2009). In contrast, tolerance to heat was not affected by hypercapnia in osmoregulating *Necora puber* (Small et al. 2010). The data indicate that crustacean species not actively regulating acid-base disturbances are most threatened by a concomitant increase of seawater CO_2 and temperature. Furthermore, growth in crustaceans, more specifically moulting, can also be compromised by a low extracellular pH as the $CaCO_3$ formation takes place in the shell fluid compartment at 0.3 to 0.5 pH units above that of the haemolymph and a decrease in haemolymph pH will increase the pH gradient between the haemolymph and the shell fluid.

Vice versa, the ability to tolerate acid-base disturbances might be an advantage to deal with high seawater CO_2. Acid-base compensatory mechanisms are accomplished via active transmembrane ion exchange and are a trade-off between acid–base balance and ion-regulation and maintenance of haemolymph ion composition. Elevated levels of haemolymph magnesium during compensation of acid-base disturbances were connected to reduced oxygen consumption during hypercapnic exposure (Small et al. 2010). Furthermore, extra- and intracellular ion regulation and the establishment of ion gradients is highly energy consuming and leads to shifts in the animals' energy budget entailing reduced somatic and reproductive growth (Stumpp et al. 2011a, b, 2012).

Whether or not the lack of compensation of hypercapnia related acid-base disturbances is an adaptive strategy or due to limited capacities, the mentioned advantages and disadvantages emphasize the complexity to evaluate CO_2 effects on marine organisms. Concluding that osmoconforming, slow-moving inactive species are the species most at risk might be premature. Certainly, the environmental variability relates to the capacities to tolerate high seawater P_{CO_2}. This especially holds true for intertidal crustacean species, which are exposed to severe fluctuations of the abiotic parameters of their environment and are strong ion- and osmoregulators (Truchot 1979). However, these species commonly experience short-term fluctuations within the supralitoral and underlying mechanisms support short-term survival in extreme environments. Special attention should be paid to long-term effects of ocean acidification. Among those species that have a sublitoral distribution and experience stable environmental conditions, the ability to compensate for acid-base disturbances is highly species dependent and it needs to be addressed, which strategies (compensation vs. non compensation) prove best on a

long-term scale. This will also have implications for species interactions and competition in future ecosystems.

It still needs to be considered that near future ecosystems will not be challenged by such high seawater CO_2 concentrations as commonly used in experiments on adult crustaceans. An increase of atmospheric PCO_2 to 970 ppm by the end of this century is projected (Caldeira and Wickett 2005), while most experiments on crustaceans were conducted between 2000 and 60000 ppm CO_2. Marine crustaceans are, thus, more tolerant towards high CO_2 levels than e.g. echinoderms and bivalves, which appear to be highly sensitive taxa negatively responding to lower increases in seawater PCO_2. Nonetheless, severe impacts of elevated seawater PCO_2 could be detected in certain early life cycle stages of *H. araneus* and other marine crustaceans implying the embryonic and larval development as potential bottlenecks in terms of ocean acidification. Therefore, future studies should aim to verify these bottlenecks at intermediate seawater CO_2 concentrations below 2000 µatm.

However, the ongoing increase of seawater PCO_2 in the ocean is occurring in synergy with increasing ocean temperature and expansion of hypoxic zones. Cumulative or interactive impact of different factors could be shown to be even more severe for osmoconforming and regulating crustaceans than CO_2 alone and the organisms' response to these multiple stressors/factors will inevitably shape the ecosystems of the future.

References

Ache BW (1982) Chemoreception and thermoreception. The Biology of Crustacea, Vol. 3 (ed. Bliss DE), pp. 369– 398, Academic Press, London

Acin-Perez R, Salazar E, Kamenetsky M, Buck J, Levin LR and Manfredi G (2009) Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation. Cell Metab 9 (3): 265-276

Anestis A, Lazou A, Pörtner HO and Michaelidis B (2007) Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. Am J Physiol Regul Integr Comp Physiol 293: R911–R921

Anger K (1983) Temperature and the larval development of *Hyas araneus* L. (Decapoda: Majidae); Extrapolation of laboratory data to field conditions. J Exp Mar Biol Ecol 69: 203-215

Anger K (1984) Development and growth in larval and juvenile *Hyas coarctatus* (Decapoda, Majidae) reared in the laboratory. Mar Ecol Prog Ser 19: 115-123

Anger K (1987) The D_0 threshold: a critical point in the larval development of decapod crustaceans. J Exp Mar Biol Ecol 108: 15-30

Anger K (2001) The biology of decapod crustacean larvae. Crustacean Issue 14. A.A. Balkema Publishers, Swets and Zeitlinger, Lisse

Anger K (2006) Contributions of larval biology to crustacean research: a review. Invertebr Reprod Dev 49 (3): 175–205

Anger K and Dawirs RR (1981) Influence of starvation on the larval development of *Hyas araneus* (Decapoda, Majidae). Helgol Meeresunters 34: 287-311

Anger K, Laasch N, Püschel C and Schorn F (1983) Changes in biomass and chemical

composition of spider crab *(Hyas araneus)* larvae reared in the laboratory. Mar Ecol Prog Ser 12: 91–101

Arnold KE, Findlay HS, Spicer JI, Daniels CL and Boothroyd D (2009) Effect of CO_2-related acidification on aspects of the larval development of the European lobster, *Homarus gammarus* (L.) Biogeosciences 6:1747-1754

Baeza JA and Fernández M (2002) Active brood care in *Cancer setosus* (Crustacea: Decapoda): the relationship between female behaviour, embryo oxygen consumption and the cost of brooding. Funct Ecol 16: 241-251

Barnhart MC and McMahon BR (1988) Depression of aerobic metabolism and intracellular pH by hypercapnia in land snails *Otala lacteal*. J Exper Biol 138: 289– 299

Bechmann RK, Taban IC, Westerlund S, Godal BF, Arnberg M, Vingen S, Ingvarsdottir A and Baussant T (2011) Effects of Ocean Acidification on early life stages of shrimp (*Pandalus borealis*) and mussel (*Mytilus edulis*). J Toxicol Environ Health, Part A, 74: 424–438

Boutilier RG, Iwama GK, Heming TA and Randall DJ (1985) The apparent pk of carbonic-acid in rainbow-trout blood-plasma between 5°C and 15°C. Resp Physiol 61: 237–254

Buckley BA, Owen ME and Hofmann GE (2001) Adjusting the thermostat: the threshold induction temperature for the heatshock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. J Exp Biol 204: 3571–3579

Caldeira K and Wickett ME (2003) Anthropogenic carbon and ocean pH. Nature 425: 365

Caldeira K and Wickett ME (2005) Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J Geophys Res-Oceans 110, C09S04

Cameron JN (1978) Effects of hypercapnia on blood acid-base status, NaCl fluxes, and trans-gill potential in freshwater blue crabs Callinectes sapidus. J Comp Physiol B 123: 137-141

Cameron JN (1985) Molting in the blue crab. Sci Am 252: 102–109

Cameron JN and Wood CN (1985) Apparent H^+ excretion and CO_2 dynamics accompanying carapace mineralization in the blue crab (*Callinectes sapidus*) following moulting. J Exp Biol 14: 181-196

Cameron JN and Iwama GK (1987) Compensation of progressive hypercapnia in channel catfish and blue crabs. J Exp Bio 133: 183-197

Cao L and Caldeira K (2008) Atmospheric CO_2 stabilization and ocean acidification. Geophys Res Lett 35: L19609

Chapman R, Mancia A, Beal M, Veloso A, Rathburn C, Blair A, Holland AF, Warr GW, Didinato G, Sokolova IM, Wirth EF, Duffy E and Sanger D (2011) The transcriptomic responses of the eastern oyster, *Crassostrea virginica*, to environmental conditions. Mol Ecol 20 (7): 1431-1449

Charmantier G (1998): Ontogeny of osmoregulation in crustaceans: a review. Invertebr Reprod Dev 33 (2-3): 177-190

Charmantier G and Charmantier-Daures M (2001) Ontogeny of osmoregulation in crustaceans: The embryonic phase. Amer Zool 41: 1078-1089

Charmantier G, Giménez L, Charmantier-Daures M and Anger K (2002) Ontogeny of osmoregulation, physiological plasticity, and larval export strategy in the grapsid crab *Chasmagnathus granulata* (Crustacea, Decapoda). Mar Ecol Prog Ser 229: 185–194

Christiansen ME (1969) Crustacea Decapoda Brachyura. Marine invertebrates of Scandinavia Vol 2. Universitetsforlaget. Oslo, Norway

Claiborne JB and Heisler N (1986) Acid-base regulation and ion transfers in the carp (*Cyprinus carpio*): pH compensation during graded long- and short-term environmental hypercapnia, and the effect of bicarbonate infusion. J Exp Biol 126: 41-61

Cummings V, Hewitt J, Van Rooyen A, Currie K, Beard S, Thrush S, Norkko J, Barr N, Heath P, Halliday NJ, Sedcole R, Gomez A, McGraw C and Metcalf V (2011) Ocean acidification at high latitudes: potential effects on functioning of the antarctic bivalve *Laternula elliptica*.

PLoS ONE 6 (1): 1-11

Dawirs RR (1983) Respiration, energy balance and development during growth and starvation of *Carcinus maenas* L. larvae (Decapoda: Portunidae). J Exp Mar Biol Ecol 69: 105-128

Deigweiher K, Koschnick N, Pörtner HO and Lucassen M (2008) Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. Am J Physiol Regul Integr Comp Physiol 295: R1660–R1670

Deigweiher K, Hirse T, Bock C, Lucassen M and Pörtner HO (2010) Hypercapnia induced shifts in gill energy budgets of Antarctic notothenioids. J Comp Physiol 180: 347–359

de la Haye KL, Spicer JI, Widdicombe S and Briffa M (2012) Reduced pH sea water disrupts chemo-responsive behaviour in an intertidal crustacean. J Exp Mar Biol Ecol 412: 134-140

de Vries MC, Rittschof R and Forward RB Jr (1991) Chemical mediation of larvae release behaviours in the crab *Neopanope sayi*. Biol Bull 180: 1–11

Dupont S, Havenhand J, Thorndyke W, Peck L and Thorndyke M (2008) Near-future level of CO_2-driven ocean acidification radically affects larval survival and development in the brittlestar *Ophiothrix fragilis*. Mar Ecol Prog Ser 373: 285–294

Dupont S, Dorey N, Stumpp M, Melzner F and Thorndyke M (2012) Long-term and trans-life-cycle effects of exposure to ocean acidification in the green sea urchin *Strongylocentrotus droebachiensis*. Mar Biol DOI 10.1007/s00227-012-1921-x

Egilsdottir H, Spicer JI and Rundle SD (2009) The effect of CO_2 acidified seawater and reduced salinity on aspects of the embryonic development of the amphipod *Echinogammarus marinus* (Leach). Mar Pollut Bull 58: 1187–1191

Etheridge DM, Steele LP, Langenfelds RL, Francey RJ, Barnola JM and Morgan VI (1998) Historical CO_2 records from the Law Dome DE08, DE08-2, and DSS ice cores. In Trends: A compendium of data on global change. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tenn, USA

Fernández M, Bock C and Pörtner HO (2000) The cost of being a caring mother: the ignored factor in the reproduction of marine invertebrates. Ecol Lett 3: 487-494

Fernández M, Ruiz-Tagle N, Cifuentes S, Pörtner HO and Arntz W (2003) Oxygen-dependent asynchrony of embryonic development in embryo masses of brachyuran crabs. Mar Biol 142: 559–565

Franke A and Clemmesen C (2011) Effect of ocean acidification on early life stages of Atlantic herring (*Clupea harengus* L.). Biogeosciences 8: 3697–3707

Frederich M and Pörtner HO (2000) Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*. Am J Physiol 279: R1531-R1538

Freire CA, Onken H and McNamara JC (2008) Structure–function analysis of ion transport in crustacean gills and excretory organs. Comp Bioch Phys A 151: 272–304

Frommel AY, Maneja R, Lowe D, Malzahn AM, Geffen AJ, Folkvord A, Piatkowski U, Reusch TBH and Clemmesen C (2012) Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. Nature Clim Change 2: 42-46

Gibbs A and Somero GN (1990) Na^+-K^+-Adenosine triphosphatase activities in gills of marine teleost fishes – changes with depths, size and locomotory acitvitiy level. Mar Biol 106: 315–321

Gutowska MA and Melzner F (2009): Abiotic conditions in cephalopod (*Sepia officinalis*) eggs: embryonic development at low pH and high pCO_2. Mar Biol 156: 515–519

Gutowska MA, Melzner F, Pörtner HO and Meier S (2010a) Cuttlebone calcification increases during exposure to elevated seawater pCO_2 in the cephalopod *Sepia officinalis*. Mar Biol 157: 1653–1663

Gutowska MA, Melzner F, Langenbuch M, Bock C, Claireaux G and Pörtner HO (2010b) Acid–base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. J Comp Physiol B 180: 323–335

Hamdoun AM, Cheney DP and Cherr GN (2003) Plasticity of HSP70 and HSP70 gene expression in the Pacific Oyster (*Crassostrea gigas*): Implications for thermal limits and induction of thermal tolerance. Biol Bull 205: 160–169

Havenhand JN , Buttler FR, Thorndyke MC and Williamson JE (2008) Near-future levels of ocean acidification reduce fertilization success in a sea urchin. Current biology 18 (15): R651–R652

Hegerl GC and Bindoff NL (2005) Warming the world´s oceans. Science 309: 254-255

Henry RP and Cameron JN (1983) The role of carbonic-anhydrase in respiration, ion regulation and acid-base-balance in the aquatic crab *Callinectes-sapidus* and the terrestrial crab *Gecarcinuslateralis*. J Exp Biol 103: 205–223

Henry RP and Wheatly MG (1992) Interaction of respiration, ion regulation, and acid-base balance in the everyday life of aquatic crustaceans. J Amer Zool 32: 407-416

Hu MY, Tseng YC, Stumpp M, Gutowska MA, Kiko R, Lucassen M and Melzner F (2011) Elevated seawater PCO_2 differentially affects branchial acid-base transporters over the course of development in the cephalopod *Sepia officinalis*. Am J Physiol Regul Integr Comp Physiol 300: R1100–R1114

Intanai I, Taylor EW and Whiteley NM (2009) Effects of salinity on rates of protein synthesis and oxygen uptake in the postlarvae and juveniles of the tropical prawn *Macrobrachium rosenbergii* (de Man). Comp Biochem Physiol A 152: 372–378

IPCC (2001) The third assessment report of the Intergovernmental Panel on Climate Change (IPCC). Cambridge University Press, Cambridge, Uk and New York, USA

IPCC (2007) The fourth assessment report of the Intergovernmental Panel on Climate Change (IPCC). Cambridge University Press, Cambridge, UK and New York, USA

Jokumsen A, Wells RMG, Ellerton HD and Weber RE (1981) Haemocyanin of the giant Antarctic isopod, *Glyptonotus antarcticus*: Structure and effects of temperature and pH on its oxygen affinity. Comp Biochem Physiol 70A: 91-95

Jost JA, Podolski SM and Frederich M (2012) Enhancing thermal tolerance by eliminating the pejus range: a comparative study with three decapod crustaceans. Mar Ecol-Prog Ser 444: 263-274

Keppel EA, Scrosati RA and Courtenay SC (2012) Ocean acidification decreases growth and development in American lobster (*Homarus americanus*) larvae. J Northw Atl Fish Sci 44: 61-66

Klein Breteler WCM (1975) Oxygen consumption and respiratory levels of juvenile shore crabs, *Carcinus maenas*, in relation to weight and temperature. Neth J Sea Res 9 (3-4): 243-254

Kurihara H (2008) Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. Mar Ecol Prog Ser 373: 275–284

Kurihara H and Ishimatsu A (2008) Effects of high CO_2 seawater on the copepod (Acartia tsuensis) through all life stages and subsequent generations. Mar Pollut Bull 56(6): 1086-1090

Kurihara H, Shimode S and Shirayama Y (2004) Effects of raised CO_2 concentration on the egg production rate and early development of two marine copepods (*Acartia steueri* and *Acartia erythraea*). Mar Pollut Bull 49: 721–727

Lallier F and Truchot JP (1989) Haemolymph oxygen transport during environmental hypoxia in the shore crab, *Carcinus maenas*. Resp Physiol 77: 323-336

Langenbuch M and Pörtner HO (2002) Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia: identifying effective acid–base variables. J Exp Biol 205: 1153–1160

Langenbuch M and Pörtner HO (2003) Energy budget of hepatocytes from Antarctic fish (*Pachycara brachycephalum* and *Lepidonotothen kempi*) as a function of ambient CO_2: pH-dependent limitations of cellular protein biosynthese? J Exp Biol 206: 3895-3903

Lannig G, Eilers S, Pörtner HO, Sokolova IM and Bock C (2010) Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas*—Changes in metabolic

pathways and thermal response. Mar Drugs 8: 2318-2339

Lenfant C and Aucutt C (1966) Measurement of blood gases by gas chromatography. Resp Physiol 1: 398–407

Lucu C and Flik G (1999) Na^+-K^+-ATPase and Na^+/Ca^+ exchange activities in gills of hyperregulating *Carcinus maenas*. Am J Physiol Regul Integr Comp Physiol 276: R490-R499

Luquet CM and Ansaldo M (1997) Acid-base balance and ion regulation during emersion in estuarine intertidal crab *Chasmagnathus granulata* Dana (Decapoda Grapsidea). Comp Biochem Phys A 3: 407-410

Luquet G and Marin F (2004) Biomineralisations in crustaceans: storage strategies. C R P 3:515–534

Martin JW and Davis GE (2006) Historical trends in crustacean systematics. Crustaceana 79: 1347–1368

Martin S, Richier S, Pedrotti ML, Castejon C, Gerakis Y, Kerros ME, Oberhänsli F, Teyssié JL, Jeffree R and Gattuso JP (2011) Early development and molecular plasticity in the Mediterranean sea urchin *Paracentrotus lividus* exposed to CO_2-driven acidification. J Exp Biol 214: 1357-1368

Meinshausen M, Smith SJ, Calvin K, Daniel JS, Kainuma MLT, Lamarque JF, Matsumoto K, Montzka SA, Raper SCB, Riahi K, Thomson A, Velders GJM and van Vuuren DPP (2011) The RCP greenhouse gas concentrations and their extensions from 1765 to 2300. Climate Change 109 (1-2): 213-241

Melzner F, Göbel S, Langenbuch M, Gutowska MA, Pörtner HO and Lucassen M (2009a) Swimming performance in Atlantic Cod (*Gadus morhua*) following long-term (4–12 months) acclimation to elevated seawater PCO_2. Aquat Toxicol 92: 30–37

Melzner F, Gutowska MA, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M and Pörtner HO (2009b) Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6: 2313–2331

Metzger R, Sartoris FJ, Langenbuch M and Pörtner HO (2007) Influence of elevated CO_2 concentrations on thermal tolerance of the edible crab *Cancer pagurus*. J Therm Biol 32: 144-151

Michaelidis B, Ouzounis C, Paleras A and Pörtner HO (2005) Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar Ecol Prog Ser 293: 109–118

Moss R et al. (2008) Towards new scenarios for analysis of emmisions, climate change, impacts, and response strategies. Technical Summary, ed Intergovernmental Panel on Climate Change (Geneva), p 25

Munday PL, Crawley NE and Nilsson GE (2009a) Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes. Mar Ecol Prog Ser Vol. 388: 235–242

Munday PL, Dixson DL, Donelson JM, Jones GP, Pratchett MS, Devitsina GV and Døving KB (2009b) Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. PNAS 106 (6): 1848-1852

O'Donnell MJ, Hammond LM and Hofmann GE (2009) Predicted impact of ocean acidification on a marine invertebrate: elevated CO_2 alters response to thermal stress in sea urchin larvae. Mar Biol 156: 439-446

Pane EF and Barry JP (2007) Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. Mar Ecol Prog Ser 334: 1-9

Pansch C, Nasrolahi A, Appelhans YS and Wahl M (2012) Impacts of ocean warming and acidification on the larval development of the barnacle *Amphibalanus improvisus*. J Exp Mar Biol Ecol 420: 48-55

Parker LM, Ross PM and O' Connor WA (2009) The effect of ocean acidification and temperature on the fertilization and embryonic development of the Sydney rock oyster *Saccostrea glomerata* (Gould 1850). Glob Change Biol 15: 2123–2136

Pelster B (2002) Developmental plasticity in the cardiovascular system of fish, with special reference to the zebra fish. Comp Biochem Physiol 133 A: 547–553

Petersen S (1995) The embryonic development of *Hyas araneus* L. (Decapoda, Majidae): Effects of temperature. Sarsia 80: 193-198

Petersen S and Anger K (1997) Chemical and physiological changes during the embryonic development of the spider crab, *Hyas araneus* L. (Decapoda: Majidae). Comp Biochem Physiol 117B (2): 299–306

Pörtner HO (1990a) Determination of intracellular buffer values after metabolic inhibition by fluoride and nitrilotriacetic acid. Respir Physiol 81: 275–288

Pörtner HO (1990b) An analysis of the effects of pH on oxygen binding by squid (*Illex illecebrosus*, *Loligo pealei*) haemocyanin. J Exp Biol 150: 407–424

Pörtner HO (2001) Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. Naturwissenschaften 88: 137–146

Pörtner HO (2002) Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp Biochem Phys A 132: 739–761

Pörtner HO (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. Mar Ecol Prog Ser 373: 203–217

Pörtner HO (2010) Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J Exp Biol 213: 881-893

Pörtner HO and Zielinski S (1998) Environmental constraints and the physiology of performance in squids. In: Payne AIL, Lipinski MR, Clarke MR, Roeleveld MAC (eds) Cephalopod biodiversity, ecology and evolution. S Afr J Mar Sci 20: 207–221

Pörtner HO and Farrell AP (2008) Physiology and climate change. Science 322: 690-692

Pörtner HO, Reipschläger A and Heisler N (1998) Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. J Exp Biol 201: 43-55

Pörtner HO, van Dijk PLM and Sommer A (2000a) Levels of metabolic cold adaption: Tradeoffs in eurythermal and stenothermal ectotherms. In: Antarctic ecosystems: models for wider ecological understanding. Davison W, Howard-Williams C, Broady P (eds), Caxton Press, Christchurch, New Zealand. pp. 109-122

Pörtner HO, Bock C and Reipschläger A (2000b) Modulation of the cost of pHi regulation during metabolic depression: A 31P-NMR study in invertebrate (*Sipunculus nudus*) isolated muscle. J Exp Biol 203: 2417-2428

Pörtner HO, Langenbuch M and Michaelidis B (2005) Synergistic effects of temperature extremes, hypoxia, and increases in CO_2 on marine animals: From Earth history to global change. J Geophys Res 110, C09S10

Pörtner HO, Bennett AF, Bozinovic F, Clarke A, Lardies MA, Lucassen M, Pelster B, Schiemer F and Stillman JH (2006) Trade-offs in thermal adaptation: The need for a molecular to ecological integration. Physiol Biochem Zool 79 (2): 295-313

Pörtner HO, Dupont S, Melzner F, Storch D and Thorndyke, M (2010a) Studies of metabolic rate and other characters across life stages. In: Riebesell U, Fabry VJ, Hansson L, Gattuso JP (eds), Guide to best practices for ocean acidification research and data reporting. Publications Office of the European Union, Luxembourg, pp. 167–180

Pörtner HO, Bickmeyer U, Bleich M, Bock C, Brownlee C, Melzner F, Michaelidis B, Sartoris FJ and Storch D (2010b) Studies of acid-base status and regulation In: Riebesell U, Fabry V, Gattuso JP (eds) Guide to best practices for ocean acidification research and data reporting. Publications Office of the European Union. Luxembourg, pp 167-180

Reipschläger A and Pörtner HO (1996) Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. J Exp Biol 199: 1801–1807

Rodolfo-Metalpa R, Houlbrèque F, Tambutté É, Boisson F, Baggini C, Patti FP, Jeffree R,

Fine M, Foggo A, Gattuso JP and Hall-Spencer JM (2011) Coral and mollusc resistance to ocean acidification adversely affected by warming. Nature Clim Change 1: 308-312

Roer R and Dillaman R (1984) The structure and calcification of the crustacean cuticle. Amer Zool 24: 893-909

Sabine CL, Feely RA, Gruber N, Key RM, Lee K, Bullister JL, Wanninkhof R, Wong CS, Wallace DWR, Tilbrook B, Millero FJ, Peng TH, Kozyr A, Ono T and Rios AF (2004) The oceanic sink for anthropogenic CO_2. Science. 305**:** 367-371

Schmidt-Nielsen K (1975) Animal Physiology. Adaptation and Environment, Cambridge: Cambridge University Press, pp. 595

Schmidt M and Gnatzy W (1984) Are the funnel-canal organs the 'campaniform sensilla' of the shore crab *Carcinus maenas* (Decapoda, Crustacea)? II. Ultrastructure. Cell Tissue Res 237: 81–93

Sedlacek C (2008) The biochemical composition of nauplii derived from stored non-diapause and diapause copepod eggs and the biology of diapausing eggs. Electronic Theses, Treatises and Dissertations. Paper 283

Sick H and Gersonde K (1969) Method for continuous registration of 0_2-binding curves of hemoproteins by means of a diffusion chamber. Anal Biochem 32: 362-376

Simpson DP (1967) Regulation of renal citrate metabolism by bicarbonate ion and pH: observations in tissue slices and mitochondria. J Clin Invest 46(2): 225

Small D, Calosi P, White D, Spicer JI and Widdicombe S (2010) Impact of medium-term exposure to CO_2 enriched seawater on the physiological functions of the velvet swimming crab *Necora puber*. Aquat Biol 10: 11–21

Soleimani M, Bookstein C, Singh G, Rao MC, Chang EB and Bastani B (1995) Differential regulation of Na+/H+ exchange and H+-ATPase by pH and HCO3- in kidney proximal tubules. J Membr Biol 144: 209–216

Somero GN (1981) pH-temperature interactions on proteins: principles of optimal pH and

buffer system design. Mar Biol Lett 2: 163-178

Spicer JI (1995) Ontogeny of respiratory function in crustaceans exhibiting either direct or indirect development. J Exp Zool 272: 413–418

Spicer JI and Eriksson SP (2003) Does the development of respiratory regulation always accompany the transition from pelagic larvae to benthic fossorial postlarvae in the Norway lobster *Nephrops norvegicus* (L.)? J Exp Mar Biol Ecol 295: 219-243

Spicer JI, Raffo A and Widdicombe S (2007) Influence of CO_2-related seawater acidification on extracellular acid-base balance in the velvet swimming crab *Necora puber*. Mar Biol 151: 1117-1125

Storch D, Santelices P, Barria J, Cabeza K, Pörtner HO and Fernández M (2009) Thermal tolerance of crustacean larvae (zoea I) in two different populations of the kelp crab *Taliepus dentatus* (Milne-Edwards). J Exp Biol 212: 1371-1376

Storch D, Fernández M, Navarrete SA and Pörtner HO (2011) Thermal tolerance of larval stages of the Chilean kelp crab *Taliepus dentatus.* Mar Ecol Prog Ser 429: 157–167

Strobel A, Bennecke S, Leo E, Mintenbeck K, Pörtner HO and Mark FC (2012) Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2. Front Zool 9 (28) doi:10.1186/1742-9994-9-28

Stumpp M, Wren J, Melzner F, Thorndyke MC and Dupont ST (2011a) CO_2 induced seawater acidification impacts sea urchin larval development I: Elevated metabolic rates decrease scope for growth and induce developmental delay. Comp Biochem Phys A 160: 331-340

Stumpp M, Dupont S, Thorndyke MC and Melzner F (2011b) CO_2 induced seawater acidification impacts sea urchin larval development II: Gene expression patterns in pluteus larvae. Comp Biochem Phys A 160: 320-330

Stumpp M, Trübenbach K, Brennecke D, Hu MY and Melzner F (2012) Resource allocation and extracellular acid–base status in the sea urchin *Strongylocentrotus droebachiensis* in response to CO_2 induced seawater acidification. Aquat Toxicol 110-111: 194-207

Svendsen H, Beszczynska-Møller A, Hagen JO, Lefauconnier B, Tverberg V, Gerland S, Ørbøk JB, Bischof K, Papucci C, Zajaczkowski M, Azzolini R, Bruland O, Wiencke C, Winther JG and Dallmann W (2002) The physical environment of Kongsfjorden— Krossfjorden, an Arctic fjord system in Svalbard. Polar Res 21: 133–166

Thomsen J and Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Mar Biol 157: 2667–2676

Thurberg FP, Dawson MA and Collier RS (1973) Effects of copper and cadmium on osmoregulation and oxygen consumption in two species of estuarine crabs. Mar Biol 23: 171-175

Todgham AE and Hofmann GE (2009) Transcriptomic response of sea urchin larvae *Strongylocentrotus purpuratus* to CO_2-driven seawater acidification. J Exp Biol 212: 2579-2594

Tomanek L and Somero GN (1999) Evolutionary and acclimation-induced variation in the heat-shock response of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. J Exp Biol 202: 2925-2936

Towle DW, Henry RP and Terwilliger NB (2011) Microarray-detected changes in gene expression in gills of green crabs (*Carcinus maenas*) upon dilution of environmental salinity. Comp Biochem Phys D 6 (2): 115-125

Tresguerres M, Parks SK, Sabatini SE, Goss GG and Luquet CM (2008) Regulation of ion transport by pH and [HCO_{3-}] in isolated gills of the crab *Neohelice* (*Chasmagnathus*) *granulate*. Am J Physiol Regul Integr Comp Physiol 294: R1033–R1043

Tresguerres M, Levin LR and Buck J (2011) Intracellular cAMP signaling by soluble adenylyl cyclase. Kidney Int 79, 1277-1288

Truchot JP (1979) Mechanisms of the compensation of blood respiratory acid-base disturbances in the shore crab, *Carcinus maenas* (L.). J Exp Zool 210 (3): 407–416

Truchot JP (1978) Blood acid-base changes during experimental emersion and reimmersion of the intertidal crab *Carcinus maenas* (L.). Respir Physiol 23: 351-360

Truchot JP (1984) Water carbonate alkalinity as a determinant of haemolymph acid–base balance in the shore crab, *Carcinus maenas*: a study at two different ambient PCO_2 and PO_2 levels. J Comp Physiol B 154: 601–606

van der Meer DLM, van den Thillart GEEJM, Witte F, de Bakker MAG, Besser J, Richardson MK, Spaink, HP, Leito JTD and Bagowski CP (2005) Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. Am J Physiol Regul Integr Comp Physiol 289: R1512–R1519

Varley DG and Greenaway P (1992) The effect of emersion on haemolymph acid-base balance and oxygen levels in *Scylla serrata* Forskal (Brachyura: Portunidae). J Exp Mar Biol Ecol 163 (1): 1-12

Walther K, Sartoris FJ, Bock C and Pörtner HO (2009) Impact of anthropogenic ocean acidification on thermal tolerance of the spider crab *Hyas araneus*. Biogeosciences 6: 2207-2215

Walther K, Anger K and Pörtner HO (2010) Effects of ocean acidification and warming on the larval development of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°N). Mar Ecol Prog Ser 417: 159-170

Walther K, Sartoris FJ and Pörtner HO (2011) Impacts of temperature and acidification on larval calcium incorporation of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°N). Mar Biol 158: 2043-2053

Weber RE and Hagerman L (1981) Oxygen and carbon dioxide transporting qualities of haemocyanin in the haemolymph of a natant decapod *Palaemon* adspersus. J Comp Physiol 145: 21 27

Wheatly MG and Henry RP (1992) Extracellular and intracellular acid-base regulation in crustaceans. J Exp Zool 263: 127-142

Whiteley NM (2011) Physiological and ecological responses of crustaceans to ocean acidification. Mar Ecol Prog Ser 430: 257–271

Whiteley NM and Taylor EW (1992) Oxygen and acid–base disturbances in the haemolymph of the lobster *Homarus gammarus* during commercial transport and storage. J Crustac Biol 12: 19–30

Wiltshire KH and Manly BFJ (2004) The warming trend at Helgoland Roads, North Sea: phytoplankton response. Helgoland Mar Res 58: 269–273

Wood H, Spicer JI and Widdicombe S (2008) Ocean acidification may increase calcification rates, but at a cost. P Roy Soc B-Biol Sci 275: 1767–1773

Zeebe RE and Wolf-Gladrow D (2001) CO_2 in seawater: equilibrium, kinetics, isotopes. Elsevier Oceanography Series, Amsterdam

Zhang D, Li S, Wang G and Guo D (2001) Impacts of CO_2-driven seawater acidification on survival, egg production rate and hatching success of four marine copepods. Acta Oceanol Sin 30 (6): 86-94

Zittier ZMC, Hirse T and Pörtner HO (2012) The synergistic effects of increasing temperature and CO_2 levels on activity capacity and acid–base balance in the spider crab, *Hyas araneus*. Mar Biol DOI 10.1007/s00227-012-2073-8

Danksagung

Ich möchte mich bei allen bedanken, die mir diese Arbeit ermöglicht und daran mitgewirkt haben, insbesondere:

Prof Hans-O. Pörtner, in dessen Arbeitsgruppe am AWI diese Arbeit entstanden ist, für die gute wissenschaftliche Betreuung und seine Diskussionsbereitschaft sowie das „Entflöhen'' der Papiere.

PD Dr Holger Auel und Prof Dr Wilhelm Hagen für die Übernahme der Begutachtung dieser Arbeit und die Bereitschaft als Gutachter und Prüfer in meinem Promotionskolloquium aufzutreten.

Dany für eine hervorragende Betreuung, sowohl wissenschaftlich als auch emotional. Du hast mich unterstützt und motiviert und konntest immer nachvollziehen wie anstrengend ein Leben als Larvenmama ist. Ich bin sehr froh, dass ich deine erste Doktorandin sein durfte.

Felix für eine super Betreuung, auch wenn es statt Haemocyanin Bindungskurven eher Larven Respiration zu diskutieren gab. Danke für die Zeit, die du dir immer nimmst, um Probleme zu besprechen und sie mit Keksen zu versüßen.

Corni für drei lustige Jahre mit viel Magnum auf der Dachterrasse, langen Abenden im Inkubationslabor und gemeinsamen Ausflügen in die Welt (oder eben nach Helgoland). Danke fürs Zuhören und Lösen der kleinen bis großen Katastrophen und die hervorragende medizinische (homöopathische!) Beratung.

Der Allergikeressensgruppe für drei Histamin-, Gluten- und Paprikafreie Jahre mit mehr oder weniger motivierten Mitgliedern, aber jeder Menge leckerem Essen.

Der gesamten IEP Arbeitsgruppe für eine tolle Zeit mit viel Spaß, besonders während der gemeinsamen Weihnachtsfeiern und Kohltouren. Insbesondere möchte ich mich bei **Timo** für seine Hilfe im Labor bedanken.

Dem Stillman Lab für die herzliche Aufnahme in ihrem Labor und eine der schönsten Erfahrungen, die ich bislang machen durfte.

Meinen zahlreichen HiWis für die tatkräftige Unterstützung bei der Larvenaufzucht.

Meinem roten AWI Anzug ohne den ich die ersten Monate am AWI nicht überstanden hätte.

Meiner Familie für die Unterstützung und den Rückhalt bei meinen Entscheidungen, die ich den letzten Jahren getroffen habe...obwohl sie immer noch nicht ganz verstanden haben, was ich da eigentlich so den ganzen Tag mache.

Lars. Ich bin froh, dass wir nicht nur privat ein tolles Team sind sondern auch allen gezeigt haben, dass man es dazu auch noch drei Jahre in einem Büro zusammen aushält. Danke für deine Liebe und Unterstützung und ein ganz besonderes Geschenk am Ende der Doktorarbeit.

Name: _____ Ort, Datum: _____

Anschrift: _____

ERKLÄRUNG

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

_____

_____

_____

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

_____
(Unterschrift)